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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 39/00, 39/395, C07K 1/00 — A1

(11) International Publication Number: **WO 97/04801**

(43) International Publication Date: 13 February 1997 (13.02.97)

(21) International Application Number: PCT/US96/12251

(22) International Filing Date: 23 July 1996 (23.07.96)

(30) Priority Data:

08/508,014	27 July 1995 (27.07.95)	US
08/615,369	14 March 1996 (14.03.96)	US

(71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).

(72) Inventors: ANDYA, James; Apartment D, 227 Richmond Drive, Millbrae, CA 94030 (US). CLELAND, Jeffrey, L.; 844 Cordilleras Avenue, San Carlos, CA 94070 (US). HSU, Chung, C.; 13120 Delson Court, Los Altos Hills, CA 94022 (US). LAM, Xanthe, M.; 280 Denslowe Drive, San Francisco, CA 94132 (US). OVERCASHIER, David, E.; 130 Vallejo Street, El Granada, CA 94018 (US). SHIRE, Steven, J.; 2417 Lincoln Avenue, Belmont, CA 94002 (US). YANG, Janet, Yu-Feng; 1860 Dale Avenue, San Mateo, CA 94401 (US). WU, Sylvia, Sau-Yan; 1438 Filbert Street #203, San Francisco, CA 94109 (US).

(74) Agents: LEE, Wendy, M. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: STABILE ISOTONIC LYOPHILIZED PROTEIN FORMULATION

(57) Abstract

A stable lyophilized protein formulation is described which can be reconstituted with a suitable diluent to generate a high protein concentration reconstituted formulation which is suitable for subcutaneous administration. For example, anti-IgE and anti-HER2 antibody formulations have been prepared by lyophilizing these antibodies in the presence of a lyoprotectant. The lyophilized mixture thus formed is reconstituted to a high protein concentration without apparent loss of stability of the protein.

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Stabile isotonic lyophilized protein formulation

Background of the Invention

Field of the Invention

This invention is directed to a lyophilized protein formulation. In particular, it relates to a stable lyophilized protein formulation which can be reconstituted with a diluent to generate a stable reconstituted formulation suitable for subcutaneous administration.

Description of Related Disclosures

In the past ten years, advances in biotechnology have made it possible to produce a variety of proteins for pharmaceutical applications using recombinant DNA techniques. Because proteins are larger and more complex than traditional organic and inorganic drugs (*i.e.* possessing multiple functional groups in addition to complex three-dimensional structures), the formulation of such proteins poses special problems. For a protein to remain biologically active, a formulation must preserve intact the conformational integrity of at least a core sequence of the protein's amino acids while at the same time protecting the protein's multiple functional groups from degradation. Degradation pathways for proteins can involve chemical instability (*i.e.* any process which involves modification of the protein by bond formation or cleavage resulting in a new chemical entity) or physical instability (*i.e.* changes in the higher order structure of the protein). Chemical instability can result from deamidation, racemization, hydrolysis, oxidation, beta elimination or disulfide exchange. Physical instability can result from denaturation, aggregation, precipitation or adsorption, for example. The three most common protein degradation pathways are protein aggregation, deamidation and oxidation. Cleland *et al. Critical Reviews in Therapeutic Drug Carrier Systems* 10(4): 307-377 (1993).

Freeze-drying is a commonly employed technique for preserving proteins which serves to remove water from the protein preparation of interest. Freeze-drying, or lyophilization, is a process by which the material to be dried is first frozen and then the ice or frozen solvent is removed by sublimation in a vacuum environment. An excipient may be included in pre-lyophilized formulations to enhance stability during the freeze-drying process and/or to improve stability of the lyophilized product upon storage. Pikal, M. *Biopharm.* 3(9)26-30 (1990) and Arakawa *et al. Pharm. Res.* 8(3):285-291 (1991).

It is an object of the present invention to provide a lyophilized protein formulation which is stable upon storage and delivery. It is a further object to provide a stable reconstituted protein formulation which is suitable for subcutaneous administration. In certain embodiments, it is an object to provide a multi-use formulation which is stable for at least the time over which it will be administered to a patient.

Summary of the Invention

This invention is based on the discovery that a stable lyophilized protein formulation can be prepared using a lyoprotectant (preferably a sugar such as sucrose or trehalose), which lyophilized formulation can be reconstituted to generate a stable reconstituted formulation having a protein concentration which is significantly higher (*e.g.* from about 2-40 times higher, preferably 3-10 times higher and most preferably 3-6 times higher) than the protein concentration in the pre-lyophilized formulation. In particular, while the protein concentration in the pre-lyophilized formulation may be 5 mg/mL or less, the protein concentration in the reconstituted formulation is generally 50 mg/mL or more. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where the formulation is intended for subcutaneous administration.

Despite the very high protein concentration in the reconstituted formulation, it has been found that the reconstituted formulation is stable (*i.e.* fails to display significant or unacceptable levels of chemical or physical instability of the protein) at 2-8°C for at least about 30 days. In certain embodiments, the reconstituted formulation is isotonic. In spite of the use of lower concentrations of the lyoprotectant to achieve such isotonic formulations upon reconstitution, it was discovered herein that the protein in the lyophilized formulation essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

When reconstituted with a diluent comprising a preservative (such as bacteriostatic water for injection, BWFI), the reconstituted formulation may be used as a multi-use formulation. Such a formulation is useful, for example, where the patient requires frequent subcutaneous administrations of the protein to treat a chronic medical condition. The advantage of a multi-use formulation is that it facilitates ease of use for the patient, reduces waste by allowing complete use of vial contents, and results in a significant cost savings for the manufacturer since several doses are packaged in a single vial (lower filling and shipping costs).

Based on the observations described herein, in one aspect the invention provides a stable isotonic reconstituted formulation comprising a protein in an amount of at least about 50 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of a protein and a lyoprotectant, wherein the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

In another embodiment, the invention provides a stable reconstituted formulation comprising an antibody in an amount of at least about 50 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of an antibody and a lyoprotectant, wherein the antibody concentration in the reconstituted formulation is about 2-40 times greater than the antibody concentration in the mixture before lyophilization.

The ratio of lyoprotectant:protein in the lyophilized formulation of the preceding paragraphs depends, for example, on both the protein and lyoprotectant of choice, as well as the desired protein concentration and isotonicity of the reconstituted formulation. In the case of a full length antibody (as the protein) and trehalose or sucrose (as the lyoprotectant) for generating a high protein concentration isotonic reconstituted formulation, the ratio may, for example, be about 100-1500 mole trehalose or sucrose:1 mole antibody.

Generally, the pre-lyophilized formulation of the protein and lyoprotectant will further include a buffer which provides the formulation at a suitable pH, depending on the protein in the formulation. For this purpose, it has been found to be desirable to use a histidine buffer in that, as demonstrated below, this appears to have lyoprotective properties.

The formulation may further include a surfactant (*e.g.* a polysorbate) in that it has been observed herein that this can reduce aggregation of the reconstituted protein and/or reduce the formation of particulates in the reconstituted formulation. The surfactant can be added to the pre-lyophilized formulation, the lyophilized formulation and/or the reconstituted formulation (but preferably the pre-lyophilized formulation) as desired.

The invention further provides a method for preparing a stable isotonic reconstituted formulation comprising reconstituting a lyophilized mixture of a protein and a lyoprotectant in a diluent such that the protein concentration in the reconstituted formulation is at least 50 mg/mL, wherein the protein concentration in the

reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

In yet a further embodiment, the invention provides a method for preparing a formulation comprising the steps of: (a) lyophilizing a mixture of a protein and a lyoprotectant; and (b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable and has a protein concentration of at least about 50 mg/mL. For example, the protein concentration in the reconstituted formulation may be from about 80 mg/mL to about 300 mg/mL. Generally, the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

An article of manufacture is also provided herein which comprises: (a) a container which holds a lyophilized mixture of a protein and a lyoprotectant; and (b) instructions for reconstituting the lyophilized mixture with a diluent to a protein concentration in the reconstituted formulation of at least about 50 mg/mL. The article of manufacture may further comprise a second container which holds a diluent (*e.g.* bacteriostatic water for injection (BWFI) comprising an aromatic alcohol).

The invention further provides a method for treating a mammal comprising administering a therapeutically effective amount of a reconstituted formulation disclosed herein to a mammal, wherein the mammal has a disorder requiring treatment with the protein in the formulation. For example, the formulation may be administered subcutaneously.

One useful anti-HER2 antibody pre-lyophilized formulation as discovered in the experiments detailed below was found to comprise anti-HER2 in amount from about 5-40 mg/mL (*e.g.* 20-30 mg/mL) and sucrose or trehalose in an amount from about 10-100 mM (*e.g.* 40-80 mM), a buffer (*e.g.* histidine, pH 6 or succinate, pH 5) and a surfactant (*e.g.* a polysorbate). The lyophilized formulation was found to be stable at 40°C for at least 3 months and stable at 30°C for at least 6 months. This anti-HER2 formulation can be reconstituted with a diluent to generate a formulation suitable for intravenous administration comprising anti-HER2 in an amount from about 10-30 mg/mL which is stable at 2-8°C for at least about 30 days. Where higher concentrations of the anti-HER2 antibody are desired (for example where subcutaneous delivery of the antibody is the intended mode of administration to the patient), the lyophilized formulation may be reconstituted to yield a stable reconstituted formulation having a protein concentration of 50 mg/mL or more.

One desirable anti-IgE antibody pre-lyophilized formulation discovered herein has anti-IgE in amount from about 5-40 mg/mL (*e.g.* 20-30 mg/mL) and sucrose or trehalose in an amount from about 60-300 mM (*e.g.* 80-170 mM), a buffer (preferably histidine, pH 6) and a surfactant (such as a polysorbate). The lyophilized anti-IgE formulation is stable at 30°C for at least 1 year. This formulation can be reconstituted to yield a formulation comprising anti-IgE in an amount from about 15-45 mg/mL (*e.g.* 15-25 mg/mL) suitable for intravenous administration which is stable at 2-8°C for at least 1 year. Alternatively, where higher concentrations of anti-IgE in the formulation are desired, the lyophilized formulation can be reconstituted in order to generate a stable formulation having an anti-IgE concentration of ≥50 mg/mL.

Brief Description of the Drawings

Figure 1 shows the effect of reconstitution volume on the stability of lyophilized rhuMAb HER2. The lyophilized formulation was prepared from a pre-lyophilization formulation comprising 25 mg/mL protein, 60

mM trehalose, 5 mM sodium succinate, pH 5.0, and 0.01% Tween 20™. The lyophilized cake was incubated at 40°C and then reconstituted with 4.0 (○) or 20.0 mL (●) of BWFI. The fraction of intact protein in the reconstituted formulation was measured by native size exclusion chromatography and defined as the peak area of the native protein relative to the total peak area including aggregates.

Figure 2 illustrates the effect of trehalose concentration on the stability of lyophilized rhuMAb HER2. The protein was lyophilized at 25 mg/mL in 5 mM sodium succinate, pH 5.0 (circles) or 5 mM histidine, pH 6.0 (squares) and trehalose concentrations ranging from 60 mM (360 molar ratio) to 200 mM (1200 molar ratio). The lyophilized protein was incubated at 40°C for either 30 days (closed symbols) or 91 days (open symbols). The amount of intact protein was measured after reconstitution of the lyophilized protein with 20 mL BWFI.

Figure 3 demonstrates the effect of trehalose concentration on the long term stability of lyophilized rhuMAb HER2 stored at 40°C. The protein was lyophilized at either 25 mg/mL in 5 mM sodium succinate, pH 5.0, 0.01% Tween 20™, and 60 mM trehalose (■) or 5 mM histidine, pH 6.0, 0.01% Tween 20™, and 60 mM trehalose (□) or 21 mg/mL in 10 mM sodium succinate, pH 5.0, 0.2% Tween 20™ and 250 mM trehalose (●). The lyophilized protein was incubated at 40°C and then reconstituted with 20 mL of BWFI. The amount of intact protein was measured after reconstitution.

Figure 4 shows the stability of rhuMAb HER2 lyophilized in 38.4 mM mannitol (7 mg/mL), 20.4 mM sucrose (7 mg/mL), 5 mM histidine, pH 6.0, 0.01% Tween 20™. The lyophilized protein was incubated at 40°C and then reconstituted with either 4.0 mL (○) or 20 mL (●) of BWFI. The amount of intact protein was measured after reconstitution.

Figure 5 demonstrates stability of reconstituted rhuMAb HER2 lyophilized in 5 mM sodium succinate, pH 5.0, 60 mM trehalose, 0.01% Tween 20™. Samples were reconstituted with either 4.0 mL (squares) or 20.0 mL (circles) of BWFI (20 mL:0.9% benzyl alcohol; 4 mL:1.1% benzyl alcohol) and then stored at 5°C (solid symbols) or 25°C (open symbols). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

Figure 6 shows stability of reconstituted rhuMAb HER2 lyophilized in 5 mM histidine, pH 6.0, 60 mM trehalose, 0.01% Tween 20. Samples were reconstituted with either 4.0 mL (squares) or 20.0 mL (circles) of BWFI (20 mL:0.9% benzyl alcohol; 4 mL:1.1% benzyl alcohol) and then stored at 5°C (solid symbols) or 25 °C (open symbols). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

Figure 7 reveals stability of reconstituted rhuMAb HER2 lyophilized in 5 mM histidine, pH 6.0, 38.4 mM mannitol, 20.4 mM sucrose, 0.01% Tween 20. Samples were reconstituted with either 4.0 mL (squares) or 20.0 mL (circles) of BWFI (20 mL:0.9% benzyl alcohol; 4 mL:1.1% benzyl alcohol) and then stored at 5°C (solid symbols) or 25 °C (open symbols). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

Figure 8 shows stability of reconstituted rhuMAb HER2 lyophilized in 10 mM sodium succinate, pH 5.0, 250 mM trehalose, 0.2% Tween 20. Samples were reconstituted with 20.0 mL of BWFI (0.9% benzyl alcohol) and then stored at 5°C (●) or 25 °C (○). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

Figure 9 shows aggregation of rhuMAb E25 formulated into buffers ranging from pH 5 to pH 7 at 10 mM buffer concentration and 5 mg/mL antibody concentration. Samples were lyophilized and assayed at time zero and after 4 weeks, 8 weeks, and 52 weeks of storage at 2-8° C. The buffers were: potassium phosphate pH 7.0 (○); sodium phosphate pH 7.0 (□); histidine pH 7.0 (◇); sodium succinate pH 6.5 (●); sodium succinate pH 6.0 (■); sodium succinate pH 5.5 (♦); and sodium succinate pH 5.0 (▲).

Figure 10 depicts aggregation of rhuMAb E25 lyophilized in 5 mM histidine buffer at both pH 6 and pH 7 and assayed following storage as follows. The buffer was at: pH 6.0 stored at 2-8° C (○); pH 6 stored at 25° C (□); pH 6 stored at 40° C (◇); pH 7 stored at 2-8° C (●); pH 7 stored at 25° C (■); and pH 7 stored at 40° C (♦).

Figure 11 illustrates aggregation of 5 mg/mL rhuMAb E25 formulated into 10 mM sodium succinate at pH 5.0 with lyoprotectant added at a concentration of 275 mM (isotonic). The lyoprotectants were: control, no lyoprotectant (○); mannitol (□); lactose (◇); maltose (●); trehalose (■); and sucrose (♦). Samples were lyophilized and assayed at time zero and after 4 weeks, 8 weeks, and 52 weeks of storage at 2-8° C.

Figure 12 shows aggregation of 5 mg/mL rhuMAb E25 formulated into 10 mM sodium succinate at pH 5.0 with lyoprotectant added at a concentration of 275 mM (isotonic). The lyoprotectants were: control, no lyoprotectant (○); mannitol (□); lactose (◇); maltose (●); trehalose (■); and sucrose (♦). Samples were lyophilized and assayed at time zero and after 4 weeks, 8 weeks, and 52 weeks of storage at 40° C.

Figure 13 depicts hydrophobic interaction chromatography of 20 mg/mL rhuMAb E25 lyophilized in histidine buffer at pH 6 with an isotonic concentration (*i.e.* 275 mM) of lactose stored for 24 weeks at 2-8, 25 or 40° C and reconstituted to 20 mg/mL.

Figure 14 shows hydrophobic interaction chromatography of 20 mg/mL rhuMAb E25 lyophilized in histidine buffer at pH 6 stored for 24 weeks at 2-8, 25 or 40° C and reconstituted to 20 mg/mL.

Figure 15 illustrates hydrophobic interaction chromatography of 20 mg/mL rhuMAb E25 lyophilized in histidine buffer at pH 6 with an isotonic concentration (*i.e.* 275 mM) of sucrose and stored for 24 weeks at 2-8, 25 or 40° C and reconstituted to 20 mg/mL.

Figure 16 illustrates the effect of sugar concentration on rhuMAb E25 formulated at 20 mg/mL in 5 mM histidine at pH 6.0. Sucrose (●) and trehalose (□) were added to the formulation at molar ratios ranging from 0 to 2010 (isotonic) (see Table 1 below). Samples were lyophilized and assayed after 12 weeks of storage at 50° C.

TABLE 1

Moles of Sugar: E25 antibody	Sugar conc. (mM)
0	0
260	34.4
380	51.6
510	68.8
760	103.1
1020	137.5
1530	206.3

2010	275

Figure 17 reveals aggregation of rhuMAb E25 formulated at 25 mg/mL into 5 mM histidine at pH 6 with 85 mM sucrose (○); 85 mM trehalose (□); 161 mM sucrose (♦) or 161 mM trehalose (▲). Samples were lyophilized and stored at 2-8°C followed by reconstitution with 0.9% benzyl alcohol to 100 mg/mL antibody in 20 mM histidine at pH 6 with isotonic (340 mM) and hypertonic (644 mM) sugar concentration.

Figure 18 shows aggregation of rhuMAb E25 formulated at 25 mg/mL into 5 mM histidine at pH 6 with 85 mM sucrose (○); 85 mM trehalose (□); 161 mM sucrose (♦) or 161 mM trehalose (▲). Samples were lyophilized and stored at 30°C followed by reconstitution with 0.9% benzyl alcohol to 100 mg/mL antibody in 20 mM histidine at pH 6 with isotonic (340 mM) and hypertonic (644 mM) sugar concentration.

Figure 19 illustrates aggregation of rhuMAb E25 formulated at 25 mg/mL into 5 mM histidine at pH 6 with 85 mM sucrose (○); 85 mM trehalose (□); 161 mM sucrose (♦) or 161 mM trehalose (▲). Samples were lyophilized and stored at 50°C followed by reconstitution with 0.9% benzyl alcohol to 100 mg/mL antibody in 20 mM histidine at pH 6 with isotonic (340 mM) and hypertonic (644 mM) sugar concentration.

Detailed Description of the Preferred Embodiments

I. Definitions

By "protein" is meant a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD.

Examples of proteins encompassed within the definition herein include mammalian proteins, such as, *e.g.*, growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), *e.g.*, M-CSF, GM-CSF, and G-CSF; interleukins (ILs), *e.g.*, IL-1 to IL-10; superoxide

dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides.

The protein which is formulated is preferably essentially pure and desirably essentially homogeneous (*i.e.* free from contaminating proteins etc). "Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition.

In certain embodiments, the protein is an antibody. The antibody may bind to any of the above-mentioned molecules, for example. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mol, p150,95, VLA-4, ICAM-1. VCAM and αv/β3 integrin including either α or β subunits thereof (*e.g.* anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; protein C etc.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules, as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, *Nature*, 256: 495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from

another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison *et al., Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature*, 321:522-525 (1986); Reichmann *et al., Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). The humanized antibody includes a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

A "stable" formulation is one in which the protein therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in *Peptide and Protein Drug Delivery*, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, New York, Pubs. (1991) and Jones, A. *Adv. Drug Delivery Rev.* 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For rapid screening, the formulation may be kept at 40°C for 2 weeks to 1 month, at which time stability is measured. Where the formulation is to be stored at 2-8°C, generally the formulation should be stable at 30°C or 40°C for at least 1 month and/or stable at 2-8°C for at least 2 years. Where the formulation is to be stored at 30°C, generally the formulation should be stable for at least 2 years at 30°C and/or stable at 40°C for at least 6 months. For example, the extent of aggregation following lyophilization and storage can be used as an indicator of protein stability (see Examples herein). For example, a "stable" formulation may be one wherein less than about 10% and preferably less than about 5% of the protein is present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation following lyophilization and storage of the lyophilized formulation can be determined. For example, a "stable" lyophilized formulation may be one wherein the increase in aggregate in the lyophilized formulation is less than about 5% and preferably less than about 3%, when the lyophilized formulation is stored at 2-8° C for at least one year. In other embodiments, stability of the protein formulation may be measured using a biological activity assay (see, *e.g.*, Example 2 below).

A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein formulation in a diluent such that the protein is dispersed in the reconstituted formulation. The reconstituted formulation in suitable for administration (*e.g.* parenteral administration) to a patient to be treated with the protein of interest and, in certain embodiments of the invention, may be one which is suitable for subcutaneous administration.

By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

A "lyoprotectant" is a molecule which, when combined with a protein of interest, significantly prevents or reduces chemical and/or physical instability of the protein upon lyophilization and subsequent storage. Exemplary lyoprotectants include sugars such as sucrose or trehalose; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher sugar alcohols, *e.g.* glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; Pluronics; and combinations thereof. The preferred lyoprotectant is a non-reducing sugar, such as trehalose or sucrose.

The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the protein in the presence of the lyoprotecting amount of the lyoprotectant, the protein essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a reconstituted formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (*e.g.* phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

A "preservative" is a compound which can be added to the diluent to essentially reduce bacterial action in the reconstituted formulation, thus facilitating the production of a multi-use reconstituted formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. The most preferred preservative herein is benzyl alcohol.

A "bulking agent" is a compound which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake (*e.g.* facilitates the production of an essentially uniform lyophilized cake which maintains an open pore structure). Exemplary bulking agents include mannitol, glycine, polyethylene glycol and xorbitol.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

II. Modes for Carrying out the Invention

A. Protein Preparation

The protein to be formulated is prepared using techniques which are well established in the art including synthetic techniques (such as recombinant techniques and peptide synthesis or a combination of these techniques) or may be isolated from an endogenous source of the protein. In certain embodiments of the invention, the protein of choice is an antibody. Techniques for the production of antibodies follow.

(i) Polyclonal antibodies.

Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining 1 mg or 1 μg of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies.

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al., Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al., Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al., Nature*, 348:552-554 (1990). Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity

(nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, *et al.*, *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate. *(iii) Humanized and human antibodies.*

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature*, 321:522-525 (1986); Riechmann *et al.*, *Nature*, 332:323-327 (1988); Verhoeyen *et al.*, *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al.*, *J. Immunol.*, 151:2296 (1993); Chothia *et al.*, *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al.*, *J. Immnol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized

antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom *et al., J. Mol. Biol.*, 227:381 (1991); Marks *et al., J. Mol. Biol.*, 222:581-597 (1991)).

(iv) Bispecific antibodies

Bispecific antibodies (BsAbs) are antibodies that have binding specificities for at least two different epitopes. Such antibodies can be derived from full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al., Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 and in Traunecker *et al., EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in

the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published March 3, 1994. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, *Methods in Enzymology*, 121:210 (1986).

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. The following techniques can also be used for the production of bivalent antibody fragments which are not necessarily bispecific. For example, Fab' fragments recovered from *E. coli* can be chemically coupled *in vitro* to form bivalent antibodies. See, Shalaby *et al.*, *J. Exp. Med.*, 175:217-225 (1992).

Various techniques for making and isolating bivalent antibody fragments directly from recombinant cell culture have also been described. For example, bivalent heterodimers have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific/bivalent antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific/bivalent antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.*, 152:5368 (1994).

B. Preparation of the Lyophilized Formulation

After preparation of the protein of interest as described above, a "pre-lyophilized formulation" is produced. The amount of protein present in the pre-lyophilized formulation is determined taking into account the desired dose volumes, mode(s) of administration etc. Where the protein of choice is an intact antibody (such as an anti-IgE or anti-HER2 antibody), from about 2 mg/mL to about 50 mg/mL, preferably from about 5 mg/mL to about 40 mg/mL and most preferably from about 20-30 mg/mL is an exemplary starting protein concentration.

The protein is generally present in solution. For example, the protein may be present in a pH-buffered solution at a pH from about 4-8, and preferably from about 5-7. Exemplary buffers include histidine, phosphate, Tris, citrate, succinate and other organic acids. The buffer concentration can be from about 1 mM to about 20 mM, or from about 3 mM to about 15 mM, depending, for example, on the buffer and the desired isotonicity of the formulation (*e.g.* of the reconstituted formulation). The preferred buffer is histidine in that, as demonstrated below, this can have lyoprotective properties. Succinate was shown to be another useful buffer.

The lyoprotectant is added to the pre-lyophilized formulation. In preferred embodiments, the lyoprotectant is a non-reducing sugar such as sucrose or trehalose. The amount of lyoprotectant in the pre-lyophilized formulation is generally such that, upon reconstitution, the resulting formulation will be isotonic. However, hypertonic reconstituted formulations may also be suitable. In addition, the amount of lyoprotectant must not be too low such that an unacceptable amount of degradation/aggregation of the protein occurs upon lyophilization. Where the lyoprotectant is a sugar (such as sucrose or trehalose) and the protein is an antibody, exemplary lyoprotectant concentrations in the pre-lyophilized formulation are from about 10 mM to about 400 mM, and preferably from about 30 mM to about 300 mM, and most preferably from about 50 mM to about 100 mM.

The ratio of protein to lyoprotectant is selected for each protein and lyoprotectant combination. In the case of an antibody as the protein of choice and a sugar (*e.g.*, sucrose or trehalose) as the lyoprotectant for generating an isotonic reconstituted formulation with a high protein concentration, the molar ratio of lyoprotectant to antibody may be from about 100 to about 1500 moles lyoprotectant to 1 mole antibody, and preferably from about 200 to about 1000 moles of lyoprotectant to 1 mole antibody, for example from about 200 to about 600 moles of lyoprotectant to 1 mole antibody.

In preferred embodiments of the invention, it has been found to be desirable to add a surfactant to the pre-lyophilized formulation. Alternatively, or in addition, the surfactant may be added to the lyophilized formulation and/or the reconstituted formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (*e.g.* polysorbates 20 or 80); poloxamers (*e.g.* poloxamer 188); Triton; sodium dodecyl sulfate (SDS); sodium laurel sulfate: sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-betaine (*e.g.* lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate; and the MONAQUAT™ series (Mona Industries, Inc., Paterson, New Jersey), polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (*e.g.* Pluronics, PF68 etc). The amount of surfactant added is such that it reduces aggregation of the reconstituted protein and minimizes the formation of particulates after reconstitution. For example, the surfactant may be present in the pre-lyophilized formulation in an amount from about 0.001-0.5%, and preferably from about 0.005-0.05%.

In certain embodiments of the invention, a mixture of the lyoprotectant (such as sucrose or trehalose) and a bulking agent (*e.g.* mannitol or glycine) is used in the preparation of the pre-lyophilization formulation. The bulking agent may allow for the production of a uniform lyophilized cake without excessive pockets therein etc.

Other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980) may be included in the pre-lyophilized formulation (and/or the lyophilized formulation and/or the reconstituted formulation) provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include: additional buffering agents; preservatives; co-solvents; antioxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (*e.g.* Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions such as sodium.

The formulation herein may also contain more than one protein as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect the other protein. For example, it may be desirable to provide two or more antibodies which bind to the HER2 receptor or IgE in a single formulation. Furthermore, anti-HER2 and anti-VEGF antibodies may be combined in the one formulation. Such proteins are suitably present in combination in amounts that are effective for the purpose intended.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to, or following, lyophilization and reconstitution. Alternatively, sterility of the entire mixture may be accomplished by autoclaving the ingredients, except for protein, at about 120°C for about 30 minutes, for example.

After the protein, lyoprotectant and other optional components are mixed together, the formulation is lyophilized. Many different freeze-dryers are available for this purpose such as Hull50™ (Hull, USA) or GT20™ (Leybold-Heraeus, Germany) freeze-dryers. Freeze-drying is accomplished by freezing the formulation and subsequently subliming ice from the frozen content at a temperature suitable for primary drying. Under this condition, the product temperature is below the eutectic point or the collapse temperature of the formulation. Typically, the shelf temperature for the primary drying will range from about -30 to 25°C (provided the product remains frozen during primary drying) at a suitable pressure, ranging typically from about 50 to 250mTorr. The formulation, size and type of the container holding the sample (*e.g.*, glass vial) and the volume of liquid will mainly dictate the time required for drying, which can range from a few hours to several days (*e.g.* 40-60hrs). A secondary drying stage may be carried out at about 0-40°C, depending primarily on the type and size of container and the type of protein employed. However, it was found herein that a secondary drying step may not be necessary. For example, the shelf temperature throughout the entire water removal phase of lyophilization may be from about 15-30°C (*e.g.*, about 20°C). The time and pressure required for secondary drying will be that which produces a suitable lyophilized cake, dependent, *e.g.*, on the temperature and other parameters. The secondary drying time is dictated by the desired residual moisture level in the product and typically takes at least about 5 hours (*e.g.* 10-15 hours). The pressure may be the same as that employed during the primary drying step. Freeze-drying conditions can be varied depending on the formulation and vial size.

In some instances, it may be desirable to lyophilize the protein formulation in the container in which reconstitution of the protein is to be carried out in order to avoid a transfer step. The container in this instance may, for example, be a 3, 5, 10, 20, 50 or 100cc vial.

As a general proposition, lyophilization will result in a lyophilized formulation in which the moisture content thereof is less than about 5%, and preferably less than about 3%.

C. **Reconstitution of the Lyophilized Formulation**

At the desired stage, typically when it is time to administer the protein to the patient, the lyophilized formulation may be reconstituted with a diluent such that the protein concentration in the reconstituted formulation is at least 50 mg/mL, for example from about 50 mg/mL to about 400 mg/mL, more preferably from about 80 mg/mL to about 300 mg/mL, and most preferably from about 90 mg/mL to about 150 mg/mL. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where subcutaneous delivery of the reconstituted formulation is intended. However, for other routes of administration, such as intravenous administration, lower concentrations of the protein in the reconstituted formulation may be desired (for example from about 5-50 mg/mL, or from about 10-40 mg/mL protein in the reconstituted formulation). In certain embodiments, the protein concentration in the reconstituted formulation is significantly higher than that in the pre-lyophilized formulation. For example, the protein concentration in the reconstituted formulation may be about 2-40 times, preferably 3-10 times and most preferably 3-6 times (*e.g.* at least three fold or at least four fold) that of the pre-lyophilized formulation.

Reconstitution generally takes place at a temperature of about 25°C to ensure complete hydration, although other temperatures may be employed as desired. The time required for reconstitution will depend, *e.g.*, on the type of diluent, amount of excipient(s) and protein. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (*e.g.* phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. The diluent optionally contains a preservative. Exemplary preservatives have been described above, with aromatic alcohols such as benzyl or phenol alcohol being the preferred preservatives. The amount of preservative employed is determined by assessing different preservative concentrations for compatibility with the protein and preservative efficacy testing. For example, if the preservative is an aromatic alcohol (such as benzyl alcohol), it can be present in an amount from about 0.1-2.0% and preferably from about 0.5-1.5%, but most preferably about 1.0-1.2%.

Preferably, the reconstituted formulation has less than 6000 particles per vial which are ≥ 10 μm in size.

D. **Administration of the Reconstituted Formulation**

The reconstituted formulation is administered to a mammal in need of treatment with the protein, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

In preferred embodiments, the reconstituted formulation is administered to the mammal by subcutaneous (*i.e.* beneath the skin) administration. For such purposes, the formulation may be injected using a syringe. However, other devices for administration of the formulation are available such as injection devices (*e.g.* the Inject-ease™ and Genject™ devices); injector pens (such as the GenPen ™); needleless devices (*e.g.* MediJector™ and BioJector™); and subcutaneous patch delivery systems.

The appropriate dosage ("therapeutically effective amount") of the protein will depend, for example, on the condition to be treated, the severity and course of the condition, whether the protein is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the protein, the type of protein used, and the discretion of the attending physician. The protein is suitably administered to the patient at one time or over a series of treatments and may be administered to the patient at any time from

diagnosis onwards. The protein may be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

Where the protein of choice is an antibody, from about 0.1-20 mg/kg is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques.

In the case of an anti-HER2 antibody, a therapeutically effective amount of the antibody may be administered to treat or prevent cancer characterized by overexpression of the HER2 receptor. It is contemplated that a reconstituted formulation of the anti-HER2 antibody may be used to treat breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon and/or bladder cancer. For example, the anti-HER2 antibody may be used to treat ductal carcinoma *in situ* (DCIS). Exemplary dosages of the anti-HER2 antibody are in the range 1-10 mg/kg by one or more separate administrations.

Uses for an anti-IgE formulation include the treatment or prophylaxis of IgE-mediated allergic diseases, parasitic infections, interstitial cystitis and asthma, for example. Depending on the disease or disorder to be treated, a therapeutically effective amount (*e.g.* from about 1-15 mg/kg) of the anti-IgE antibody is administered to the patient.

E. Articles of Manufacture

In another embodiment of the invention, an article of manufacture is provided which contains the lyophilized formulation of the present invention and provides instructions for its reconstitution and/or use. The article of manufacture comprises a container. Suitable containers include, for example, bottles, vials (*e.g.* dual chamber vials), syringes (such as dual chamber syringes) and test tubes. The container may be formed from a variety of materials such as glass or plastic. The container holds the lyophilized formulation and the label on, or associated with, the container may indicate directions for reconstitution and/or use. For example, the label may indicate that the lyophilized formulation is reconstituted to protein concentrations as described above. The label may further indicate that the formulation is useful or intended for subcutaneous administration. The container holding the formulation may be a multi-use vial, which allows for repeat administrations (*e.g.* from 2-6 administrations) of the reconstituted formulation. The article of manufacture may further comprise a second container comprising a suitable diluent (*e.g.* BWFI). Upon mixing of the diluent and the lyophilized formulation, the final protein concentration in the reconstituted formulation will generally be at least 50 mg/mL. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All literature citations are incorporated by reference.

EXAMPLE 1

ANTI-HER2 FORMULATION

Overexpression of the *HER2* proto-oncogene product ($p185^{HER2}$) has been associated with a variety of aggressive human malignancies. The murine monoclonal antibody known as muMAb4D5 is directed against the extracellular domain (ECD) of $p185^{HER2}$. The muMAb4D5 molecule has been humanized in an attempt to improve its clinical efficacy by reducing immunogenicity and allowing it to support human effector functions

(see WO 92/22653). This example describes the development of a lyophilized formulation comprising full length humanized antibody huMAb4D5-8 described in WO 92/22653.

In the development of a lyophilized formulation, excipients and buffers are initially screened by measuring the stability of the protein after lyophilization and reconstitution. The lyophilized protein in each formulation is also subjected to accelerated stability studies to determine the potential stability of the protein over its shelf-life. These accelerated studies are usually performed at temperatures above the proposed storage conditions and the data are then used to estimate the activation energy for the degradation reactions assuming Arrhenius kinetics (Cleland *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* 10(4): 307-377 (1993)). The activation energy is then used to calculate the expected shelf-life of the protein formulation at the proposed storage conditions.

In early screening studies, the stability of several lyophilized recombinant humanized anti-HER2 antibody (rhuMAb HER2) formulations was investigated after incubation at 5° C (proposed storage condition) and 40° C (accelerated stability condition). In the liquid state, rhuMAb HER2 was observed to degrade by deamidation (30Asn of light chain) and isoaspartate formation via a cyclic imide intermediate, succinimide (102Asp of heavy chain). The deamidation was minimized at pH 5.0 resulting in degradation primarily at the succinimide. At pH 6.0, slightly greater deamidation was observed in the liquid protein formulation. The lyophilized formulations were therefore studied with: (a) 5 or 10 mM succinate buffer, pH 5.0 or (b) 5 or 10 mM histidine buffer, pH 6.0. Both buffers contained the surfactant, polysorbate 20 (Tween 20™), which was employed to reduce the potential for aggregation of the reconstituted protein and minimize the formation of particulates after reconstitution. These buffers were used with and without various sugars. The protein was formulated in the buffer at 5.0, 21.0 or 25.0 mg/mL. These formulations were then lyophilized and assessed for protein stability after 2 weeks at 5° C and 40° C. In the lyophilizer, the vials were frozen at a shelf temperature of -55° C for approximately 5 hours followed by primary drying at a shelf temperature of 5° C and 150 mTorr for 30 hours, and drying to 1-2% residual moisture was achieved with secondary drying at a shelf temperature of 20° C for 10 hours. The major degradation route for this protein upon lyophilization was aggregation, and therefore the protein stability was assessed by native size exclusion chromatography to measure the recovery of intact native protein (% intact protein in Table 2 below).

The stabilizing effects of various lyoprotectant sugars on lyophilized protein was measured in 10 mM sodium succinate, pH 5.0 (Table 2). At high sugar concentrations (250-275 mM) and low protein concentration (5.0 mg/mL), trehalose and lactose stabilized the protein against aggregation for the lyophilized protein stored for 2 weeks at 40° C. However, lactose, a reducing sugar, was observed to react with the protein over longer term storage at 40° C. The formulations at 5.0 mg/mL protein containing either sorbitol or mannitol yielded aggregated protein after storage at 40° C for 2 weeks. At the higher protein concentration (21.0 mg/mL), formulations comprising mannitol, or mannitol in combination with sorbitol or glycine, contained aggregated protein after lyophilization and storage at both conditions. In contrast, trehalose and sucrose prevented aggregation at both storage conditions.

The 250 mM trehalose and 250 mM lactose formulations were assessed for long term stability. After 9 months at 40° C or 12 months at 5° C, there was no change in the % intact protein for the trehalose formulation. For the lactose formulation, the % intact protein remained constant (same as initial) after 3 months at 40° C or

6 months at 25°C. The trehalose formulation could be stored at controlled room temperature (15-30°C) for 2 years without a significant change in % intact protein.

The 10 mM histidine, pH 6.0 formulation with mannitol contained less aggregated protein after storage at 40°C for 2 weeks than the 10 mM succinate formulation, pH 5.0 with mannitol. This result may be related to some stabilizing effect contributed by histidine alone. After storage at 40°C for 2 weeks, there was, however, significant aggregation for histidine alone or histidine/mannitol formulations. The addition of sucrose at an equal mass to mannitol (10 mg/mL of each) in the histidine formulation stabilized the protein against aggregation for both storage conditions. The use of glycine with mannitol did not improve the protein stability, while the sucrose/glycine formulation provided the same stability as the sucrose/mannitol formulation. These results further indicated that sucrose was useful for preventing aggregation of the lyophilized protein during storage.

TABLE 2

Composition Prior to Lyophilization		% Intact Protein[a]		
[Protein][b] (mg/mL)	Formulation	Liquid (5°C)	Lyophilized (2 wk, 5°C)	Lyophilized (2wk, 40°C)
	10 mM sodium succinate pH 5.0			
5.0	275 mM trehalose, 0.01% Tween 20™	98.9	99.1	98.9
5.0	275 mM lactose, 0.01% Tween 20™	96.8	96.5	96.6
5.0	275 mM sorbitol, 0.01% Tween 20™	99.4	99.3	95.4
5.0	250 mM mannitol, 0.01% Tween 20™	100.0	99.9	98.8
5.0	250 mM trehalose, 0.01% Tween 20™	100.0	99.9	100.0
5.0	250 mM lactose, 0.01% Tween 20™	100.0	100.0	100.0
21.0	250 mM trehalose, 0.2% Tween 20™	99.3	99.1	99.1
21.0	250 mM sucrose, 0.2% Tween 20™	99.6	99.6	99.7
21.0	250 mM mannitol, 0.01% Tween 20™	100.0	94.6	94.0
21.0	188 mM mannitol/63 mM sorbitol, 0.01% Tween 20™	99.8	98.6	96.5
21.0	250 mM mannitol/25 mM glycine, 0.01% Tween 20™	99.5	96.5	96.4
	10 mM histidine pH 6.0			
21.0	No sugar, 0.01% Tween 20™	100.0	99.9	98.9
21.0	54.9 mM mannitol, 0.01% Tween 20™	100.0	99.9	99.2
21.0	29.2 mM sucrose/266.4 mM glycine, 0.01% Tween 20™	100.0	100.0	99.6
21.0	54.9 mM mannitol/266.4 mM glycine, 0.01% Tween 20™	100.0	99.8	98.9

Composition Prior to Lyophilization		% Intact Protein[a]		
[Protein][b] (mg/mL)	Formulation	Liquid (5° C)	Lyophilized (2 wk, 5° C)	Lyophilized (2wk, 40° C)
21.0	54.9 mM mannitol/29.2 mM sucrose, 0.01% Tween 20™	99.8	100.0	99.7

a. The fraction of intact protein was measured by native size exclusion HPLC and the peak area of the native protein relative to the total peak area including aggregates (TSK3000 SW XL column, TosoHaas, with a flow rate of 1.0 mL/min; elution with phosphate buffered saline; detection at 214 and 280 nm). The protein formulations were analyzed before lyophilization (liquid, 5° C) and after lyophilization and storage at 5° C or 40° C for 2 weeks.
b. Formulations containing 5 mg/mL protein were reconstituted with distilled water (20 mL, 5.0 mg/mL protein), and formulations containing 21 mg/mL protein were reconstituted with bacteriostatic water for injection (BWFI, 0.9% benzyl alcohol; 20 mL, 20 mg/mL protein).

The delivery of a high protein concentration is often required for subcutaneous administration due to the volume limitations (≤ 1.5 mL) and dosing requirements (≥100 mg). However, high protein concentrations (≥ 50 mg/mL) are often difficult to achieve in the manufacturing process since at high concentrations, the protein has a tendency to aggregate during processing and becomes difficult to manipulate (*e.g.* pump) and sterile filter. Alternatively, the lyophilization process may provide a method to allow concentration of the protein. For example, the protein is filled into vials at a volume (Vf) and then lyophilized. The lyophilized protein is then reconstituted with a smaller volume (Vr) of water or preservative (*e.g.* BWFI) than the original volume (*e.g.* Vr = 0.25Vf) resulting in a higher protein concentration in the reconstituted solution. This process also results in the concentration of the buffers and excipients. For subcutaneous administration, the solution is desirably isotonic.

The amount of trehalose in the lyophilized rhuMAb HER2 was reduced to produce an isotonic solution upon reconstitution to yield 100 mg/mL protein. The stabilizing effect of trehalose was determined as a function of concentration for 5 mM sodium succinate, pH 5.0 and 5 mM histidine, pH 6.0 at 25.0 mg/mL protein (Table 3). At trehalose concentrations from 60 to 200 mM, there was no significant aggregation after incubation of the lyophilized protein for 4 weeks at 40° C. These formulations were reconstituted with 20 mL of bacteriostatic water for injection (BWFI, USP, 0.9% benzyl alcohol). Reconstitution of the 50 mM trehalose formulation (5 mM sodium succinate) with 4 mL of BWFI (100 mg/mL protein) after incubation for 4 weeks at 40° C yielded a slight increase in aggregate formation. The preserved reconstituted formulations provided the advantage of multiple withdrawals from the same vial without sterility concerns. When sterile needles are used, these formulations would then allow for several doses from a single vial.

TABLE 3

Composition Prior to Lyophilization		% Intact Protein[a]		
[Protein] (mg/mL)	Formulation	Liquid (5°C)	Lyophilized (4 wk, 5°C)	Lyophilized (4 wk, 40°C)
	5 mM sodium succinate pH 5.0			
25.0	50 mM trehalose, 0.01% Tween 20™ [b]	100.00	100.0	99.5
25.0	60 mM trehalose, 0.01% Tween 20™	100.0	100.0	99.9
25.0	60 mM trehalose, 0.01% Tween 20™	100.0	100.0	99.2
25.0	100 mM trehalose, 0.01% Tween 20™	100.0	100.0	99.7
25.0	150 mM trehalose, 0.01% Tween 20™	100.0	100.0	99.8
25.0	200 mM trehalose, 0.01% Tween 20™	100.0	100.0	100.0
	5 mM histidine pH 6.0			
25.0	38.4 mM mannitol/20.4 mM sucrose, 0.01% Tween 20™	100.0	100.0	99.3
25.0	38.4 mM mannitol/20.4 mM sucrose, 0.01% Tween 20™ [c]	100.0	100.0	99.4
25.0	60 mM trehalose, 0.01% Tween 20™ [d]	100.0	100.0	99.8
25.0	60 mM trehalose, 0.01% Tween 20™	100.0	100.0	99.4
25.0	100 mM trehalose, 0.01% Tween 20™	100.0	100.0	99.6
25.0	150 mM trehalose, 0.01% Tween 20™	100.0	100.0	100.0
25.0	200 mM trehalose, 0.01% Tween 20™	100.0	100.0	100.0

a. The fraction of intact protein was measured by native size exclusion HPLC and defined as the peak area of the native protein relative to the total peak area including aggregates (TSK3000 SW XL column, TosoHaas, with a flow rate of 1.0 mL/min; elution with phosphate buffered saline; detection at 214 and 280 nm). The protein formulations were analyzed before lyophilization (liquid, 5°C) and after lyophilization and storage at 5°C or 40°C for 4 weeks. Formulations were reconstituted with bacteriostatic water for injection (BWFI, USP, 0.9% w/w benzyl alcohol; 20 mL, 22 mg/mL protein).

b. Reconstituted with 4 mL of BWFI (0.9% benzyl alcohol) to yield 100 mg/mL protein.

c. Reconstituted with 4 mL of BWFI (1.1% benzyl alcohol) to yield 100 mg/mL protein.

d. Sample incubated for 2 weeks at 5°C or 40°C and then reconstituted with 20 mL of BWFI (0.9% benzyl alcohol) to yield 22 mg/mL protein.

Currently, rhuMAb HER2 is under investigation as a therapeutic for the treatment of breast cancer. The protein is dosed to patients at 2 mg/kg on a weekly basis. Since the average weight of these patients is 65 kg, the average weekly dose is 130 mg of rhuMAb HER2. For subcutaneous administration, injection volumes of 1.5 mL or less are well tolerated and, therefore, the protein concentration for a weekly subcutaneous administration of rhuMAb HER2 may be approximately 100 mg/mL (130 mg average dose/1.5 mL). As mentioned above, this high protein concentration is difficult to manufacture and maintain in a stable form. To achieve this high protein concentration, rhuMAb HER2 formulated in: (a) 5 mM sodium succinate, pH 5.0 or (b) 5 mM histidine, pH 6.0, was lyophilized at 25 mg/mL protein in 60 mM trehalose, 0.01% Tween 20™. The lyophilization was performed by filling 18 mL of the protein formulation into 50 cc vials. In the lyophilizer, the vials were frozen at a shelf

temperature of -55°C for approximately 5 hours followed by primary drying at a shelf temperature of 5°C and 150 mTorr for 30 hours, and drying to 1-2% residual moisture was achieved with secondary drying at a shelf temperature of 20°C for 10 hours. Thermocouples placed in vials containing the placebo (formulation without protein) indicated that the product in the vials was maintained below -10°C throughout primary drying. Sequential stoppering studies during the lyophilization revealed that the residual moisture after primary drying was usually less than 10%.

The lyophilized protein was then reconstituted with either 4 or 20 mL of BWFI (0.9 or 1.1% benzyl alcohol) to yield concentrated protein solutions:

(a) 4 mL: 102 mg/mL rhuMAb HER2, 245 mM trehalose, 21 mM sodium succinate, pH 5.0 or 21 mM histidine, pH 6.0, 0.04% Tween 20™;

(b) 20 mL: 22 mg/mL rhuMAb HER2, 52 mM trehalose, 4 mM sodium succinate, pH 5.0 or 4 mM histidine, pH 6.0, 0.009% Tween 20™.

After storage of the lyophilized formulations for 4 weeks at 40°C and reconstitution to 22 mg/mL protein, the amount of aggregated protein appeared to increase slightly with decreasing trehalose concentration. The stability of the lyophilized protein was not affected by the volume of reconstitution. As shown in Figure 1, the amount of intact protein after incubation of the lyophilized protein at 40°C was the same for the 60 mM trehalose, 5 mM sodium succinate, pH 5.0, 0.01% Tween 20™ formulation reconstituted with either 4 or 20 mL of BWFI.

The results shown in Table 3 suggested that there may be a relationship between the trehalose concentration and the protein stability. To further assess this relationship, the formulations containing different concentrations of trehalose formulated in either sodium succinate or histidine were incubated for 91 days at 40°C. The stability was then measured as a function of the trehalose to protein molar ratio for each concentration of trehalose. As shown in Figure 2, the protein stability clearly decreased with decreasing trehalose concentration for both formulations. There was no apparent difference between the two buffers, succinate and histidine, in these formulations suggesting that the primary stabilizer under these conditions is trehalose. In addition, the observed decrease in intact protein for both these formulations would be acceptable even at the low trehalose concentration for a formulation that is stored at 2-8°C throughout its shelf-life. However, if controlled room temperature (30°C maxmimum temperature) stability is required, higher trehalose concentrations (≥ 600:1 trehalose:protein) may be needed depending on the stability specifications for the product (*i.e.* the specification for the amount of intact protein remaining after 2 years of storage). Typically, a controlled room temperature storage condition would require stability for 6 months at 40°C which is equivalent to storage at 30°C for 2 years.

As shown in Figure 3, the 250 mM trehalose formulation was unchanged after 6 months at 40°C while both the 60 mM trehalose formulations were less stable. The 60 mM trehalose formulations may then require refrigerated storage if the product specification at the end of its shelf-life is, for example, >98% intact protein by native size exclusion chromatography.

In the previous screening study, sucrose was also observed to prevent aggregation of rhuMAb HER2 after lyophilization and subsequent storage. To achieve isotonic solutions after reconstitution for subcutaneous administration (approximately four-fold concentration of formulation components and protein), the sucrose concentration must be reduced significantly. The equal mass concentration of sucrose and mannitol (bulking

agent) used in the screening studies prevented aggregation of the protein. A lower concentration of sucrose and mannitol (equal mass concentrations) was chosen as a potential subcutaneous formulation of rhuMAb HER2. The protein solution (25 mg/mL protein, 5 mM histidine, pH 6.0, 38.4 mM (7 mg/mL) mannitol, 20.4 mM (7 mg/mL) sucrose, 0.01% Tween 20™) was lyophilized in the same manner as the 60 mM trehalose formulation except that the primary drying cycle was extended to 54 hours. After 4 weeks at 40°C, there was a slight increase in the amount of aggregates after reconstitution with 4.0 or 20.0 mL of BWFI (Table 3). The amount of aggregated protein was the same for reconstitution at 22 or 100 mg/mL protein (Figure 4). Like the 60 mM trehalose formulations, the mannitol/sucrose formulation yielded less intact protein over time at 40°C. The molar ratio of sucrose to protein for this formulation was 120 to 1, indicating that the mannitol/sucrose combination may be more effective than trehalose alone at the same molar ratio of stabilizing sugar (Figures 2 and 4).

In the previous examples, the stability of the lyophilized rhuMAb HER2 formulations was determined as a function of temperature. These studies demonstrated that the trehalose and mannitol/sucrose formulations prevented degradation of the protein in the lyophilized state at high temperatures (40°C). However, these experiments did not address the stability of the protein after reconstitution and storage. Once reconstituted with BWFI, the lyophilized rhuMAb HER2 formulations may be used for several administrations of the drug. In particular, the vial configuration (450 mg rhuMAb HER2) was designed to provide three doses to the average patient (130 mg rhuMAb HER2 per dose). Since the drug is dosed weekly, the vial may be stored at least three weeks after reconstitution. To assure that the rhuMAb HER2 remained stable after reconstitution, stability studies on the reconstituted rhuMAb HER2 formulations were performed at 5°C and 25°C.

For subcutaneous administration, the formulations were reconstituted to 100 mg/mL (4 mL BWFI). At this high protein concentration, the protein may be more susceptible to aggregation than the intravenous dosage form that was reconstituted to 22 mg/mL protein (20 mL BWFI). The four rhuMAb HER2 formulations from the previous example were assessed for aggregation (loss of intact protein). As shown in Tables 4 through 6, there was no difference in stability for formulations reconstituted at 22 and 100 mg/mL protein. Furthermore, these formulations maintained the protein completely intact for up to 90 days at 5 °C and 30 days at 25 °C, indicating that the reconstituted protein could be stored refrigerated for at least 90 days. Unlike the lyophilized protein stability in the previous example, the trehalose concentration in the formulation did not affect the protein stability (Table 7).

TABLE 4

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 25 mg/mL protein in 5 mM sodium succinate, pH 5.0, 60 mM trehalose, 0.01% Tween 20™

Time (days)	% Intact Protein			
	22 mg/mL protein		100 mg/mL protein	
	5°C	25°C	5°C	25°C
0	99.9	99.9	99.7	99.7
14	ND	100.0	ND	100.0
30	100.0	100.0	100.0	100.0
91	99.8	ND	100	ND

The samples were reconstituted with 4.0 or 20.0 mL of BWFI (1.1% or 0.9% benzyl alcohol), and then stored at 5°C or 25°C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

TABLE 5

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 25 mg/mL protein in 5 mM histidine, pH 6.0, 60 mM trehalose, 0.01% Tween 20™

Time (days)	% Intact Protein			
	22 mg/mL protein		100 mg/mL protein	
	5°C	25°C	5°C	25°C
0	100.0	100.0	100.0	100.0
14	ND	100.0	ND	100.0
31	99.3	99.7	100.0	100.0
61	100.0	ND	ND	ND

The samples were reconstituted with 4.0 or 20.0 mL of BWFI (1.1% or 0.9% benzyl alcohol), and then stored at 5°C or 25 °C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

TABLE 6

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 25 mg/mL protein in 5 mM histidine, pH 6.0, 38.4 mM mannitol, 20.4 mM sucrose, 0.01% Tween 20™

Time (days)	% Intact Protein			
	22 mg/mL protein		100 mg/mL protein	
	5°C	25°C	5°C	25°C
0	99.7	99.7	99.8	99.8
14	ND	100.0	ND	99.8
31	100.0	100.0	100.0	100.0
92	100.0	ND	100.0	ND

The samples were reconstituted with 4.0 or 20.0 mL of BWFI (1.1% or 0.9% benzyl alcohol), and then stored at 5°C or 25 °C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

TABLE 7

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 21 mg/mL protein in 10 mM sodium succinate, pH 5.0, 250 mM trehalose, 0.2% Tween 20™

Time (days)	% Intact Protein 21 mg/mL protein	
	5°C	25°C
0	99.8	99.8
14	ND	100.0
31	99.9	99.4
92	99.8	ND

The samples were reconstituted with 20.0 mL of BWFI (0.9% benzyl alcohol), and then stored at 5°C or 25°C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

As mentioned previously, the major degradation route for rhuMAb HER2 in aqueous solutions is deamidation or succinimide formation. The loss of native protein due to deamidation or succinimide formation was assessed for the four reconstituted rhuMAb HER2 formulations.

Analysis of rhuMAb HER2 deamidation and succinimide formation was performed using cation exchange chromatography. A Bakerbond Wide-Pore Carboxy Sulfon (CSX) column (4.6 x 250 mm) was operated at a flow rate of 1 mL/min. The mobile phase buffers were (A) 0.02 M sodium phosphate, pH 6.9, and (B) 0.02 M sodium phosphate, pH 6.9, 0.2 M NaCl. The chromatography was then performed at 40°C as follows:

TABLE 8

Time (min)	% Buffer B
0	10
55	45
57	100
62	100
62.1	10
63	10

Peak elution was monitored at 214 nm and 75 μg of protein was loaded for each analysis.

Again, there were no differences in stability of the formulations reconstituted at 22 and 100 mg/mL protein (Figures 5 through 7). The protein degradation was more rapid at 25°C than 5°C for each formulation, and the rate of degradation was comparable for all the formulations stored at 5°C. The formulations containing histidine underwent a slightly greater rate of degradation at 25°C than the succinate formulations. The amount of trehalose in the formulation did not affect the degradation rate at either temperature (Figures 5 and 8). These

results indicated that these four formulations provide an acceptable rate of degradation under refrigerated storage conditions (5°C) for the intended period of use (30 days after reconstitution with BWFI).

Multi-use formulations should pass preservative efficacy testing as described by the US Pharmacopeia (USP) for use in the United States. The rhuMAb HER2 lyophilized formulation consisting of 25 mg/mL protein, 5 mM histidine, pH 6.0, 60 mM trehalose, 0.01% Tween 20™ was reconstituted with 20 mL of benzyl alcohol at concentrations between 0.9 and 1.5% w/w. For concentrations at or above 1.3% w/w, the reconstituted solution became cloudy after overnight incubation at room temperature (~25 °C). Reconstitution with the standard BWFI solution (0.9% benzyl alcohol) resulted in a solution that did not consistently pass the preservative challenge tests. However, reconstitution with 1.0 or 1.1% benzyl alcohol was both compatible with the formulation and passed the preservative challenge testing. The manufacturer's specifications for the solution required a range of ± 10%, and therefore, the lyophilized formulations are reconstituted with 1.1% benzyl alcohol (1.1 ± 0.1%).

A single step lyophilization cycle for the rhuMAb HER2 formulation was developed . In the single step lyophilization cycle, rhuMAb HER2 at 25 mg/mL, 60 mM trehalose, 5 mM histidine pH 6 and 0.01% polysorbate 20 was lyophilized at a shelf temperature of 20° C. and a pressure of 150 mTorr. After 47 hours, the residual moisture content of the lyophilized cake was less than 5%. This lyophilization cycle is considered to be useful in that it simplifies the manufacturing process, by eliminating the secondary drying step.

EXAMPLE 2

ANTI-IgE FORMULATION

IgE antibodies bind to specific high-affinity receptors on mast cells, leading to mast cell degranulation and release of mediators, such as histamine, which produce symptoms associated with allergy. Hence, anti-IgE antibodies that block binding of IgE to its high-affinity receptor are of potential therapeutic value in the treatment of allergy. These antibodies must also not bind to IgE once it is bound to the receptor because this would trigger histamine release. This example describes the development of a lyophilized formulation comprising full length humanized anti-IgE antibody MaE11 described in Presta *et al. J. Immunology,* 151: 2623-2632 (1993).

Materials: Highly purified rhuMAb E25 (recombinant humanized anti-IgE antibody MaE11) which did not contain Tween 20™ was used in the formulations described below. Spectra/Por 7 dialysis membranes were purchased from Spectrum (Los Angeles, CA). All other reagents used in this study were obtained from commercial sources and were of analytical grade. Formulation buffers and chromatography mobile phase were prepared by mixing the appropriate amount of buffer and salt with Milli-Q water in a volumetric flask.

Formulation: E25 S Sepharose pool was dialyzed into formulation buffers as specified. Dialysis was accomplished by a minimum of 4 x 2L buffer exchanges over a 48 hour period at 2-8° C. Following dialysis, lyoprotectant was added at a isotonic concentration to some of the formulations as required. Protein concentration following dialysis was determined by UV spectroscopy using a molar absorptivity of 1.60. The dialyzed protein was diluted to the predetermined formulation concentration with an appropriate formulation buffer, sterile filtered using a 0.22 µm Millex-GV filter (Millipore) and dispensed into pre-washed and autoclaved glass vials. The vials were fitted with siliconized teflon lyophilization stoppers and lyophilized using the following conditions: the E25 formulation was frozen to -55° C at 80° C/hour and the vial content was kept frozen for 4 hours. The shelf temperature was ramped to 25° C at 10° C/hour for primary drying. Primary

drying was carried out at 25°C, 50 μ chamber vacuum pressure for 39 hours such that the residual moisture of the lyophilized cake was 1-2%. Following lyophilization, a vial of each formulation was removed for t=0 analysis and the remaining vials were held at various temperatures which include -70°C. 2-8°C. 25°C, 30°C (controlled room temperature) 40°C and 50°C.

Chromatography: Native size exclusion chromatography was carried out on a Bio-Rad Bio-Select™ SEC 250-5 column (300 x 7.8 mm). The column was equilibrated and ran in PBS at a flow rate of 0.5 mL/min using a Hewlett Packard 1090L HPLC equipped with a diode array detector. Molecular weight standards (Bio-Rad, Inc.) consisting of thyroglobulin (670 kd), gamma-globulin (158 kd), ovalbumin (44 kd), and cyanocobalamin (1.35 kd) were used to calibrate the column. The sample load was 25 μg and protein was detected by monitoring the UV absorption at 214 nm using Turbochrom 3 software (PE Nelson, Inc).

Hydrophobic Interaction Chromatography: $F(ab')_2$ fragments of the E25 antibody were chromatographed using a TosoHaas Butyl-NPR column (3.5 x 4.6 mm) and a Hewlett Packard 1090L HPLC equipped with a diode array detector. Elution buffer A was: 20 mM Tris, 2 M ammonium sulfate, 20% (v/v) glycerol, pH 8.0 while elution buffer B was: 20 mM Tris, 20% (v/v) glycerol, pH 8.0. The column was equilibrated with 10% elution buffer B at a flow rate of 1.0 mL/min for a minimum of 20 minutes. The sample load was 5 μg and protein was detected by monitoring the UV absorption at 214 nm using Turbochrom 3 data acquisition software (PE Nelson, Inc). Following injection of the sample, the column was maintained at 10% buffer B for 1 minute followed by a linear gradient of from 10% to 62% buffer B in 20 minutes. The column was washed with 100% buffer B for 5 minutes and re-equilibrated with 10% buffer B for a minimum of 20 minutes between successive sample injections.

Antibody Binding Activity: IgE receptor binding inhibition assay (IE25:2) was carried out as described in Presta *et al.*, *supra*. on samples diluted to 20 μg/mL and 30 μg/mL in assay diluent (phosphate buffered saline, 0.5% BSA, 0.05% polysorbate 20, 0.01% Thimerosol). Each dilution was then assayed in triplicate and the results were multiplied by an appropriate dilution factor to yield an active concentration. The results from 6 assays were averaged. The assay measures the ability of rhuMAb E25 to competitively bind to IgE and thereby prevent IgE from binding to its high affinity receptor which is immobilized to an ELISA plate. The results are divided by the antibody concentration as determined by UV absorption spectroscopy and reported as a specific activity. Previous experiments have shown that this assay is stability indicating.

Particulate Assay: Reconstituted vials of lyophilized rhuMAb E25 were pooled to achieve a volume of approximately 7 mL. A count of the number of particles of size ranging from 2 to 80 μm present in 1 mL of sample was determined using a Hiac/Royco model 8000 counter. The counter was first washed with 1 mL of sample three times followed by the measurement of 1 mL of sample in triplicate. The instrument determines the number of particles per mL that are equal to or greater than 10 μm and the number of particles per mL that are equal to or greater than 25 μm.

The first step in the development of a formulation for the anti-IgE antibody was to determine a suitable buffer and pH for lyophilization and storage of the product. Antibody at a concentration of 5.0 mg/mL was formulated into 10 mM succinate buffers ranging from pH 5.0 to pH 6.5 and into sodium phosphate, potassium phosphate and histidine buffers at pH 7.0. Figure 9 shows increased antibody aggregate was observed in the higher pH formulations both before and after lyophilization. An exception was the histidine formulation at pH

7, where no increase in aggregate was observed upon storage at 2-8°C. Figure 10 shows rhuMAb E25 lyophilized in 5 mM histidine buffer at both pH 6 and pH 7 and stored for 1 year at 2-8°C, 25°C, and 40°C. At each assay time point and storage temperature the pH 6 formulation had less aggregate than the antibody formulated at pH 7. These results show histidine at pH 6 is a particularly useful buffer system for preventing aggregation of the antibody.

To facilitate screening of lyoprotectants, the anti-IgE antibody was formulated into sodium succinate at pH 5 with or without a lyoprotectant. Potential lyoprotectants, added at isotonic concentrations, were grouped into 3 categories:

(a) non-reducing monosaccharide (*i.e.* mannitol);

(b) reducing disaccharides (*i.e.* lactose and maltose); and

(c) non-reducing disaccharides (*i.e.* trehalose and sucrose).

Aggregation of the formulations following storage at 2-8°C and 40°C for one year is shown in Figures 11 and 12. With storage at 2-8°C, the monosaccharide formulation (mannitol) aggregated at a rate similar to the buffer control, while formulations containing the disaccharides were equally effective in controlling aggregation (Figure 11). The results following storage at 40°C where similar with the exception of the sucrose formulation which rapidly aggregated (which correlated with a browning of the freeze-dried cake (Figure 12)). This was later shown to be caused by degradation of sucrose following storage at both acidic pH and high temperature.

Hydrophobic interaction chromatography of the antibody formulated in histidine buffer at pH 6 with lactose shows the antibody is altered following storage for 6 months at 40°C (Figure 13). The chromatography peaks are broadened and the retention time decreases. These changes are not observed with the buffer control and sucrose formulations stored under similar conditions as shown in Figures 14 and 15, respectively. Furthermore, isoelectric focusing showed an acidic shift in the pI of the antibody formulated in lactose and stored at 25°C and 40°C. This indicates that reducing sugars are not suitable as lyoprotectants for the antibody.

Aggregation of lyophilized formulations of anti-IgE at a concentration of 20 mg/mL in 5 mM histidine buffer at pH 6 with various concentrations of sucrose and trehalose following storage for 12 weeks at 50°C is shown in Figure 16. Both sugars have a similar protective effect on aggregation when the sugar concentration is greater than 500 moles of sugar per mole of antibody. From these results, isotonic and hypertonic formulations of both sucrose and trehalose were identified for further development. The formulations are designed to be filled prior to lyophilization at a relatively low concentration of antibody and the lyophilized product is reconstituted with less volume than was filled with bacteriostatic water for injection (BWFI) comprising 0.9% benzyl alcohol. This allows the concentration of the antibody immediately prior to subcutaneous delivery and includes a preservative for a potential multi-use formulation while avoiding interactions between the protein and preservative upon long-term storage.

Isotonic formulation: Anti-IgE at 25 mg/mL formulated in 5 mM histidine buffer at pH 6 with 500 moles of sugar per mole antibody which equals a sugar concentration of 85 mM. This formulation is reconstituted with BWFI (0.9% benzyl alcohol) at a volume which is four times less than was filled. This results in a 100 mg/mL of antibody in 20 mM histidine at pH 6 with an isotonic sugar concentration of 340 mM.

Hypertonic formulation: Anti-IgE at 25 mg/mL formulated in 5 mM histidine buffer at pH 6 with 1000 moles of sugar per mole antibody which equals a sugar concentration of 161 mM. This formulation is reconstituted with BWFI (0.9% benzyl alcohol) at a volume which is four times less than was filled. This results in a 100 mg/mL of antibody in 20 mM histidine at pH 6 with a hypertonic sugar concentration of 644 mM.

Comparisons of the antibody aggregation following storage of the isotonic and hypertonic formulations for up to 36 weeks are shown in Figures 17 to 19. No change in aggregation is observed in either the hypertonic or isotonic formulations with storage at 2-8°C (Figure 17). With storage at controlled room temperature (30°C) increased aggregation is not observed in the hypertonic formulations while an increase in aggregate of from 1 to 2% occurs in the isotonic formulations (Figure 18). Finally, following storage at 50°C a minimal increase in aggregate is observed with the hypertonic formulations, a 4% increase in aggregate occurs with the isotonic trehalose formulation and a 12% increase in aggregate occurs with the isotonic sucrose formulation (Figure 19). These results show the isotonic formulation contains the minimum amount of sugar necessary to maintain the stability of the antibody with storage at a temperature up to 30°C.

The binding activity of the anti-IgE in the isotonic and hypertonic formulations was measured in an IgE receptor inhibition assay. It was discovered that the binding activity of the isotonic and hypertonic sucrose and trehalose formulations was essentially unchanged following storage at -70°C, 2-8°C, 30°C and 50°C for up to 36 weeks.

Lyophilized formulations of proteins are known to contain insoluble aggregates or particulates (Cleland *et al., Critical Reviews in Therapeutic Drug Carrier Systems*, 10 (4):307-377 (1993)). Accordingly, a particulate assay of antibody lyophilized at a concentration of 25 mg/mL in 5 mM histidine, pH 6 with the addition of 85 mM and 161 mM sucrose and trehalose was performed. Polysorbate 20 was added to the formulations at a concentration of 0.005%, 0.01%, and 0.02%. Samples were lyophilized and assayed following reconstitution to 100 mg/mL antibody in 20 mM histidine, pH 6 with 340 mM and 644 mM sugar. The polysorbate 20 concentration following reconstitution was 0.02%, 0.04%, and 0.08%.

Table 9 below shows the number of particles of size equal to or greater than 10 μm and equal to or greater than 25 μm from the isotonic and hypertonic sucrose and trehalose formulations. Polysorbate 20 was added to the formulations at concentrations of 0.005%, 0.01%, and 0.02% prior to lyophilization. The results show that the addition of Tween™ to the formulation significantly reduces the number of particles in each size range tested. The US Pharmacopeia (USP) specification for small volume injections are not more than 6,000 particles of greater than or equal to 10 μm and not more than 600 particles of greater than or equal to 25 μm per container (Cleland *et al., supra*). With the addition of polysorbate 20, both the hypertonic and isotonic formulations pass this specification.

TABLE 9

Formulation	Polysorbate 20	Particles per mL	
		≥ 10 µm	≥ 25 µM
Isotonic Sucrose	None	16,122	28
	0.005%	173	2
	0.01%	224	5
	0.02%	303	6
Hypertonic Sucrose	None	14,220	84
	0.005%	73	6
	0.01%	51	0
	0.02%		6
Isotonic Trehalose	None	33,407	24
	0.005%	569	4
	0.01%	991	16
	0.02%	605	9
Hypertonic Trehalose	None	24,967	28
	0.005%	310	11
	0.01%	209	6
	0.02%	344	6

One formulation developed for the anti-IgE antibody (*i.e.* 143 mg vial isotonic formulation of rhuMAb E25) which is considered to be useful for subcutaneous delivery of this antibody is shown in Table 10 below. A 10 cc vial is filled with 5.7 mL of rhuMAb E25 at a concentration of 25 mg/mL formulated in 5 mM histidine at pH 6.0 with 0.01% polysorbate 20. Sucrose is added as a lyoprotectant at a concentration of 85 mM which corresponds to a molar ratio of sugar to antibody of 500 to 1. The vial is lyophilized and reconstituted with 0.9% benzyl alcohol to one quarter of the volume of the fill or 1.2 mL. The final concentration of components in the formulation is increased four fold to 100 mg/mL rhuMAb E25 in 20 mM histidine at pH 6 with 0.04% polysorbate 20 and 340 mM sucrose (isotonic) and 0.9% benzyl alcohol. The formulation contains histidine buffer at pH 6 because of its demonstrated protective effect on antibody aggregation. Sucrose was added as the lyoprotectant because of previous use in the pharmaceutical industry. The concentration of sugar was chosen to result in an isotonic formulation upon reconstitution. Finally, polysorbate 20 is added to prevent the formation of insoluble aggregates.

TABLE 10

Pre-lyophilized Formulation (Fill 5.7 mL into 10 cc vial)	Reconstituted Formulation (1.2 mL 0.9% Benzyl Alcohol)
25 mg/mL rhuMAb E25	100 mg/mL rhuMAb E25
5 mM Histidine, pH 6.0	20 mM Histidine, pH 6.0
85 mM Sucrose	340 mM Sucrose
0.01% Polysorbate 20	0.04% Polysorbate 20
-	0.9% Benzyl Alcohol

WHAT IS CLAIMED IS:

1. A stable isotonic reconstituted formulation comprising a protein in an amount of at least about 50 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of a protein and a lyoprotectant, wherein the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

2. The formulation of claim 1 wherein the lyoprotectant is sucrose or trechalose.

3. The formulation of claim 1 which further comprises a buffer.

4. The formulation of claim 3 wherein the buffer is histidine or succinate.

5. The formulation of claim 1 which further comprises a surfactant.

6. A stable reconstituted formulation comprising an antibody in an amount of at least about 50 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of an antibody and a lyoprotectant, wherein the antibody concentration in the reconstituted formulation is about 2-40 times greater than the antibody concentration in the mixture before lyophilization.

7. The formulation of claim 6 wherein the antibody is an anti-IgE antibody or anti-HER2 antibody.

8. The formulation of claim 6 which is isotonic.

9. A method for preparing a stable isotonic reconstituted formulation comprising reconstituting a lyophilized mixture of a protein and a lyoprotectant in a diluent such that the protein concentration in the reconstituted formulation is at least 50 mg/mL, wherein the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

10. A method for preparing a formulation comprising the steps of:

(a) lyophilizing a mixture of a protein and a lyoprotectant; and

(b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable and has a protein concentration of at least about 50 mg/mL.

11. The method of claim 10 wherein the protein concentration in the reconstituted formulation is from about 80 mg/mL to about 300 mg/mL.

12. The method of claim 10 wherein the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

13. The method of claim 10 wherein lyophilization is performed at a shelf temperature maintained at about 15-30°C throughout the entire lyophilization process.

14. An article of manufacture comprising:

(a) a container which holds a lyophilized mixture of a protein and a lyoprotectant; and

(b) instructions for reconstituting the lyophilized mixture with a diluent to a protein concentration in the reconstituted formulation of at least about 50 mg/mL.

15. The article of manufacture of claim 14 further comprising a second container which holds a diluent.

16. The article of manufacture of claim 15 wherein the diluent is bacteriostatic water for injection (BWFI) comprising an aromatic alcohol.

17. A formulation comprising a lyophilized mixture of a lyoprotectant and an antibody, wherein the molar ratio of lyoprotectant:antibody is about 100-1500 mole lyoprotectant:1 mole antibody.

18. Use of the formulation of claim 1 in the preparation of a medicament for treating a mammal which has a disorder requiring treatment with the protein in the formulation.

19. Use as in claim 18 wherein the formulation is for subcutaneous administration.

20. A formulation comprising anti-HER2 antibody in amount from about 5-40 mg/mL, sucrose or trehalose in an amount from about 10-100 mM, a buffer and a surfactant.

21. The formulation of claim 20 further comprising a bulking agent.

22. The formulation of claim 20 which is lyophilized and stable at 30°C for at least 6 months.

23. The formulation of claim 20 which is reconstituted with a diluent such that the anti-HER2 antibody concentration in the reconstituted formulation is from about 10-30 mg/mL, wherein the reconstituted formulation is stable at 2-8°C for at least about 30 days.

24. A formulation comprising anti-IgE antibody in amount from about 5-40 mg/mL, sucrose or trehalose in an amount from about 80-300 mM, a buffer and a surfactant.

25. The formulation of claim 24 which is lyophilized and stable at about 30°C for at least 1 year.

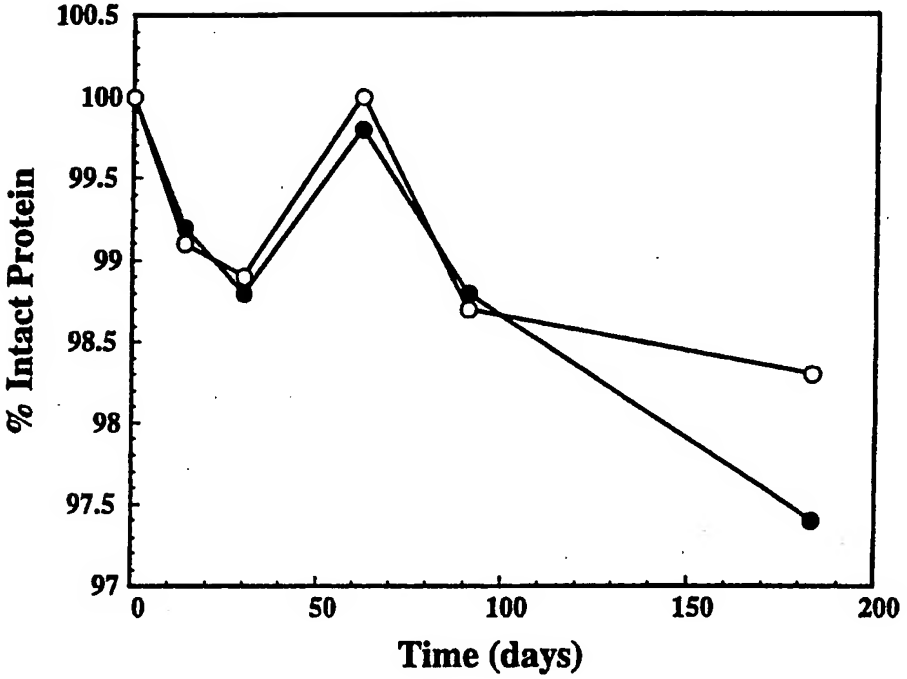
100.5
100
99.5
99
98.5
98
97.5
97
% Intact Protein
0
50
100
150
200
Time (days)

FIG. 1

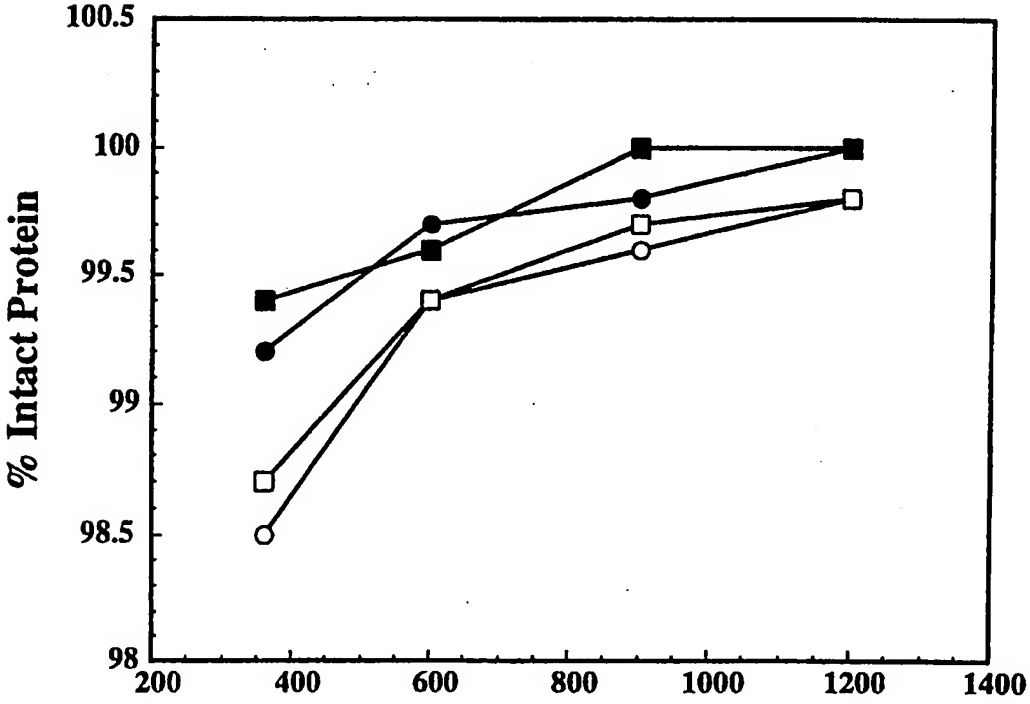
100.5
100
99.5
99
98.5
98
% Intact Protein
200
400
600
800
1000
1200
1400
[Trehalose]/[Protein] (molar ratio)

FIG. 2

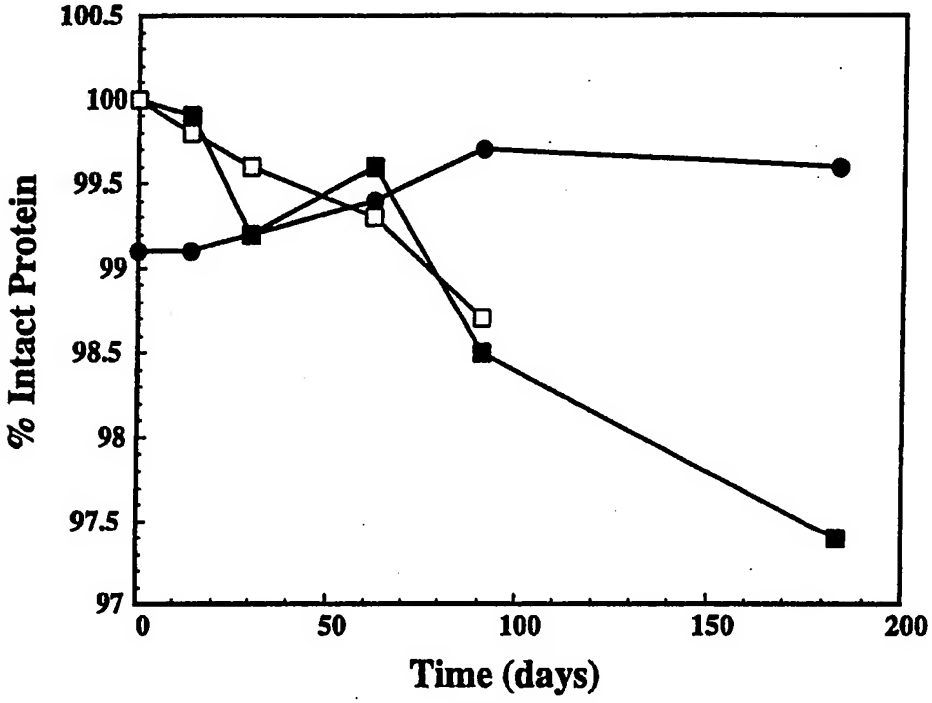
100.5
100
99.5
99
98.5
98
97.5
97
% Intact Protein
0
50
100
150
200
Time (days)

FIG. 3

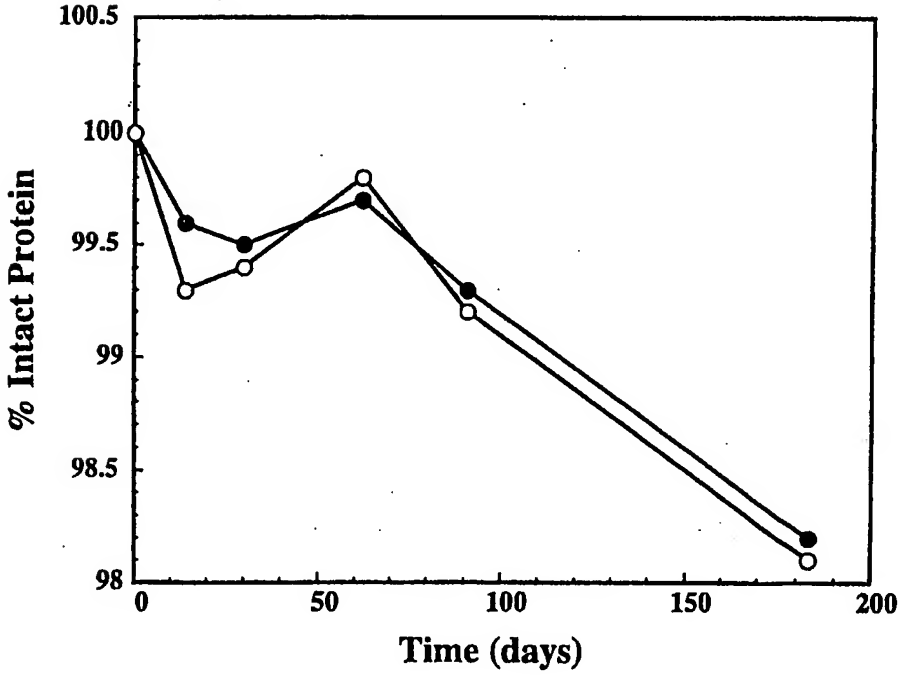
100.5
100
99.5
99
98.5
98
% Intact Protein
0
50
100
150
200
Time (days)

FIG. 4

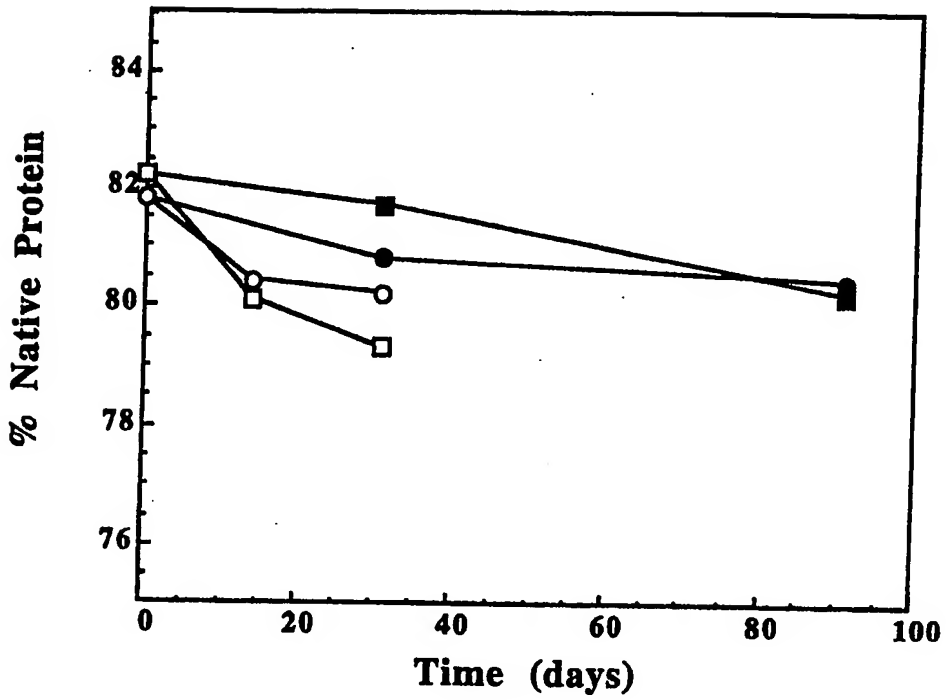
% Native Protein
84
82
80
78
76
0
20
40
60
80
100
Time (days)

FIG. 5

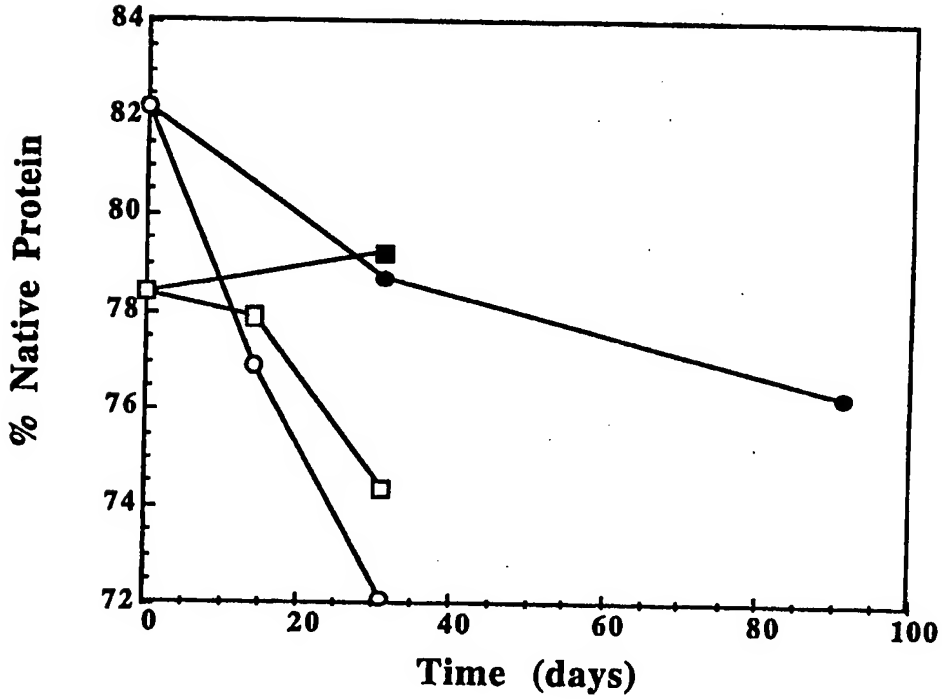
% Native Protein
84
82
80
78
76
74
72
0
20
40
60
80
100
Time (days)

FIG. 6

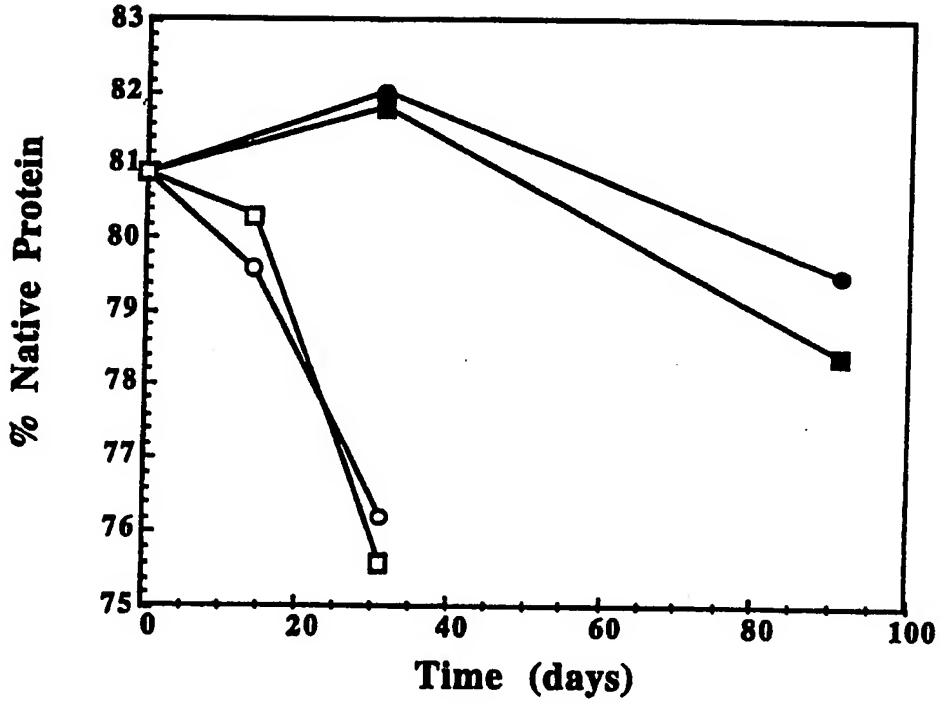
% Native Protein
83
82
81
80
79
78
77
76
75
0
20
40
60
80
100
Time (days)

FIG. 7

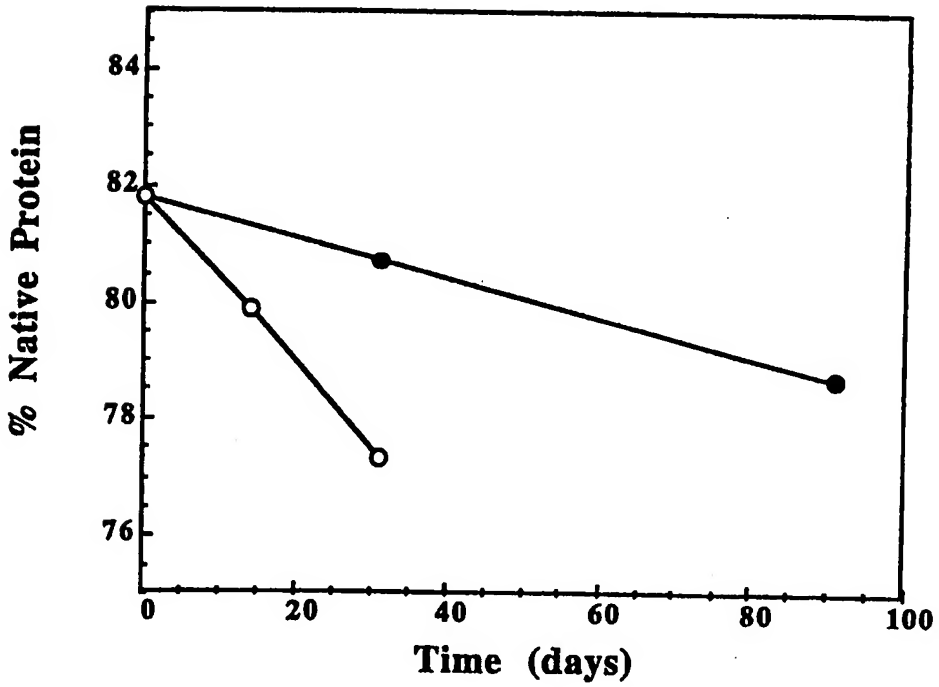
% Native Protein
84
82
80
78
76
0
20
40
60
80
100
Time (days)

FIG. 8

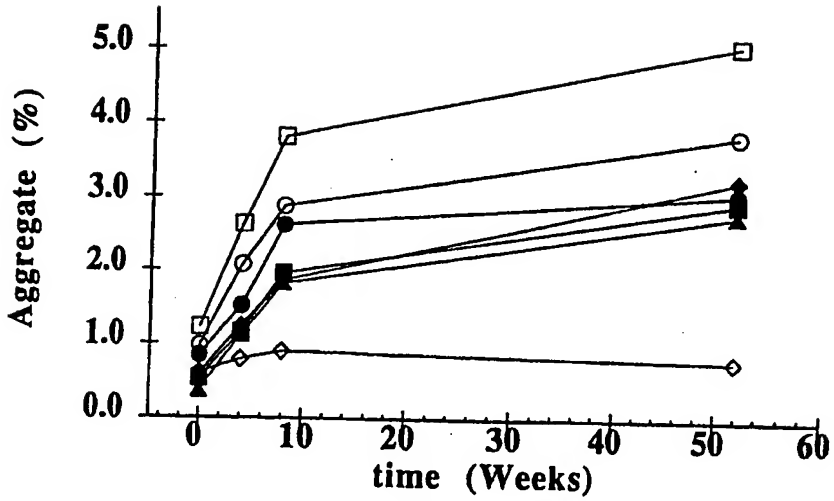
Aggregate (%)
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
50
60
time (Weeks)

FIG. 9

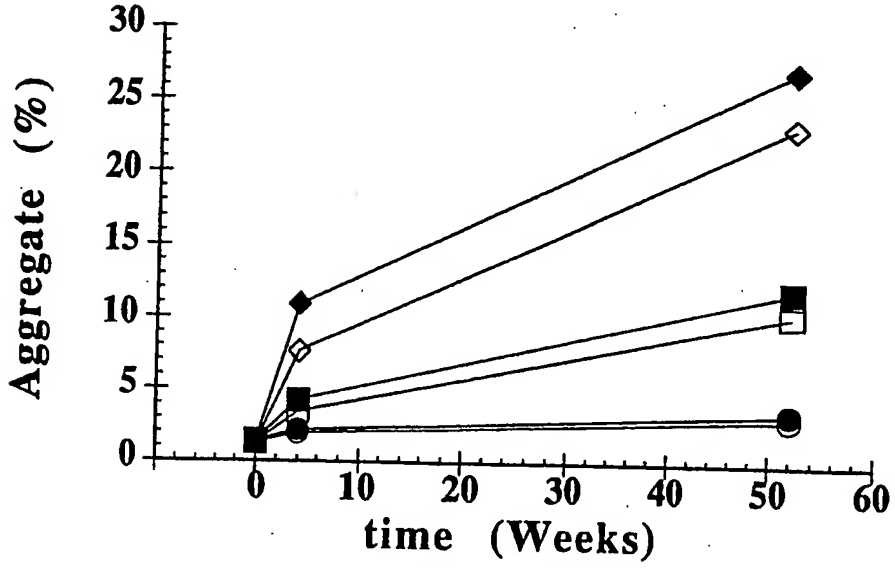
Aggregate (%)
30
25
20
15
10
5
0
0
10
20
30
40
50
60
time (Weeks)

FIG. 10

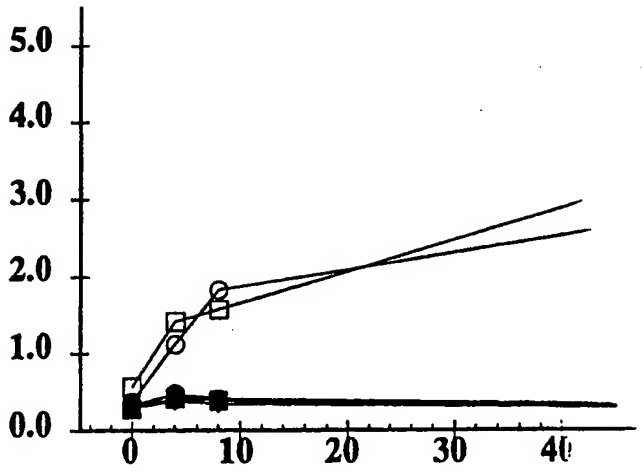

FIG. 11

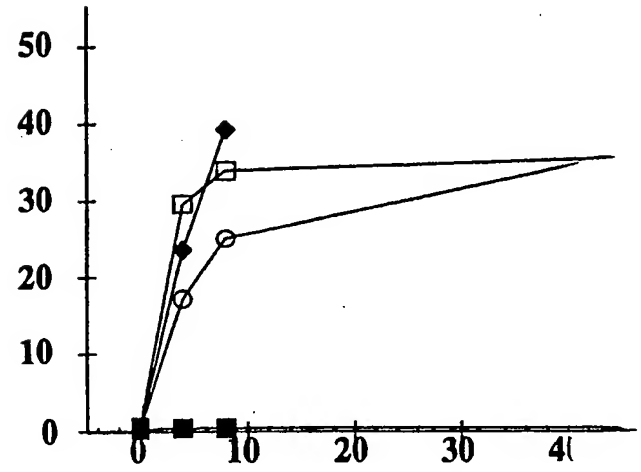

FIG. 12

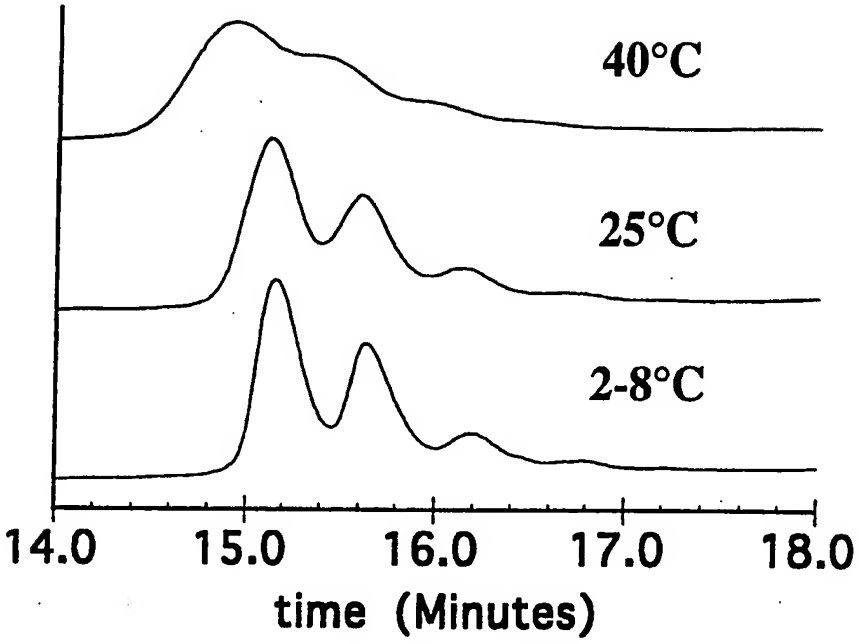
40°C
25°C
2-8°C
14.0
15.0
16.0
17.0
18.0
time (Minutes)

FIG. 13

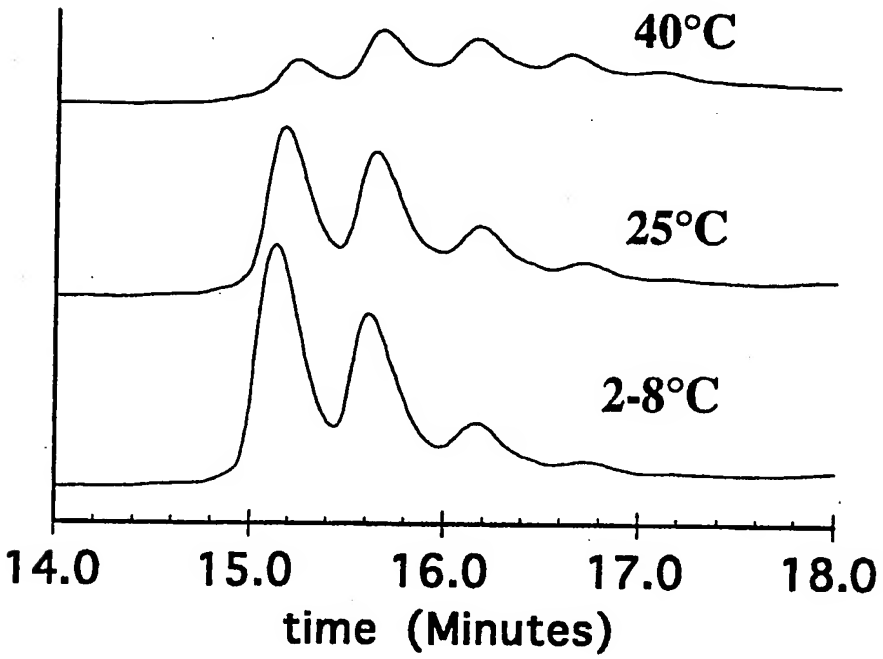
40°C
25°C
2-8°C
14.0
15.0
16.0
17.0
18.0
time (Minutes)

FIG. 14

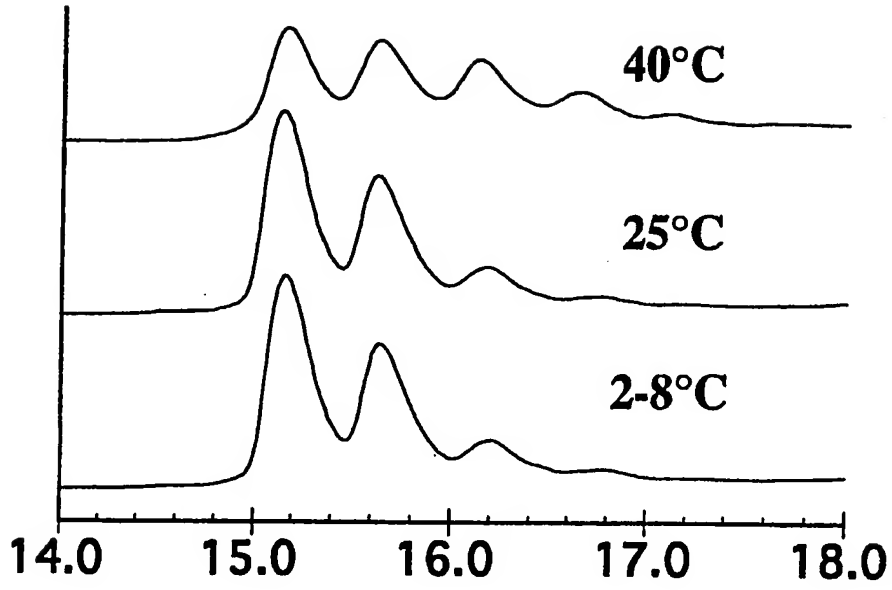
40°C
25°C
2-8°C
14.0
15.0
16.0
17.0
18.0
time (Minutes)

FIG. 15

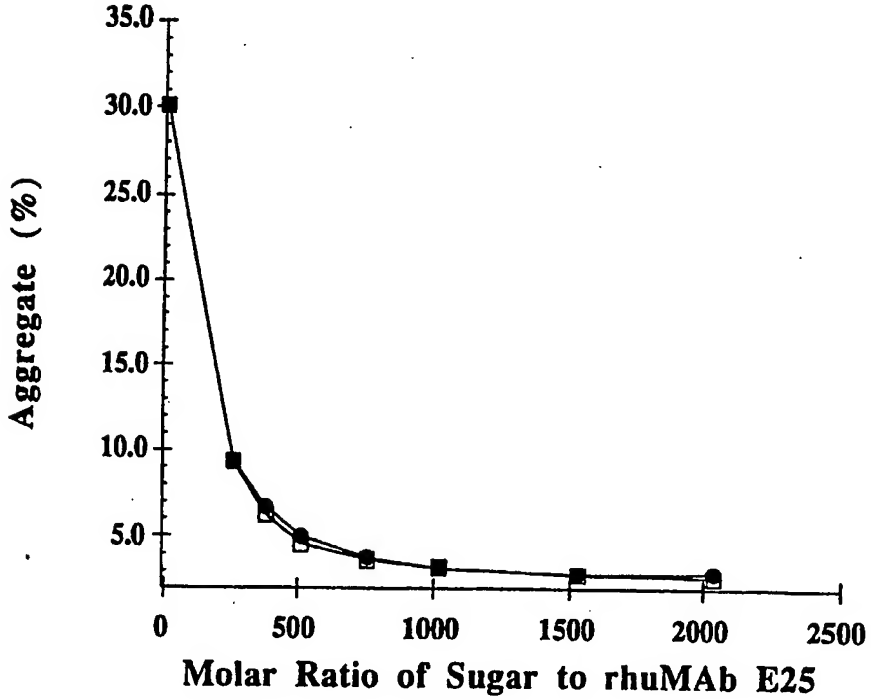
35.0
30.0
25.0
20.0
15.0
10.0
5.0
Aggregate (%)
0
500
1000
1500
2000
2500
Molar Ratio of Sugar to rhuMAb E25

FIG. 16

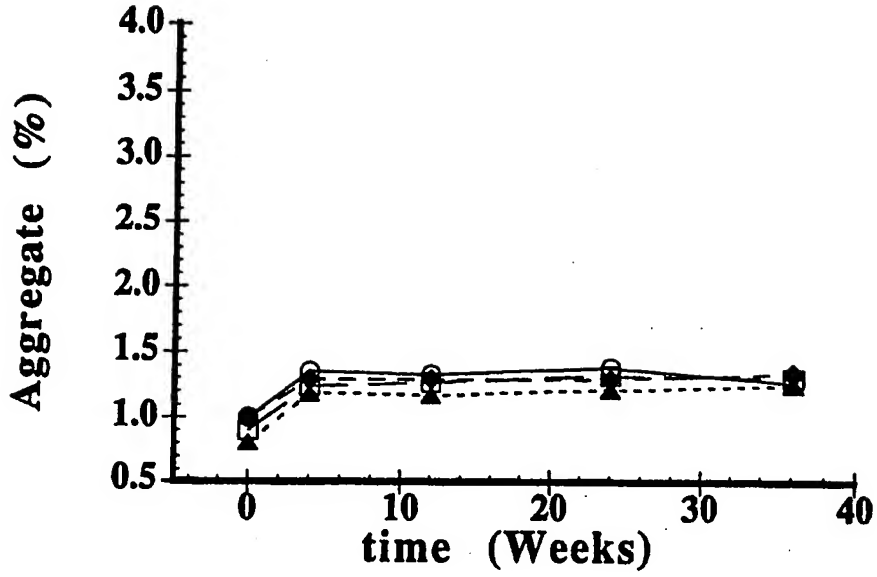
Aggregate (%)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
10
20
30
40
time (Weeks)

FIG. 17

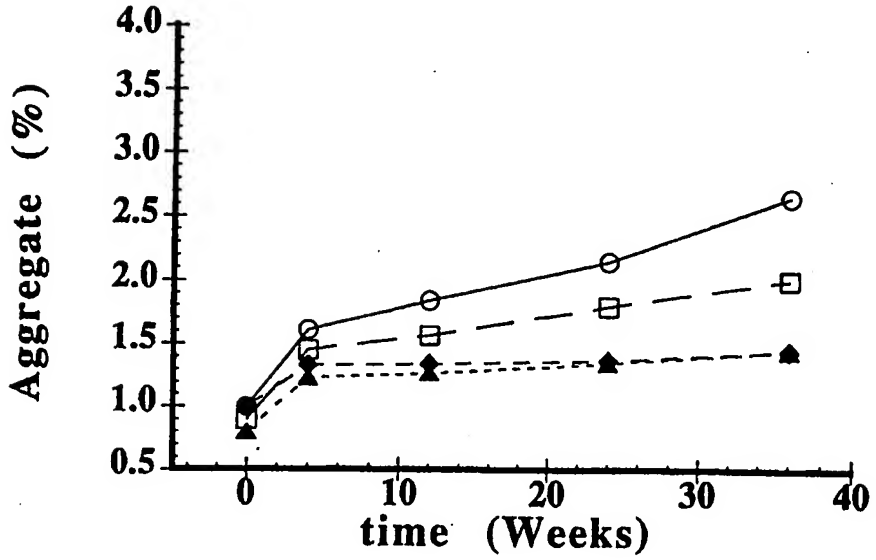
Aggregate (%)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
10
20
30
40
time (Weeks)

FIG. 18

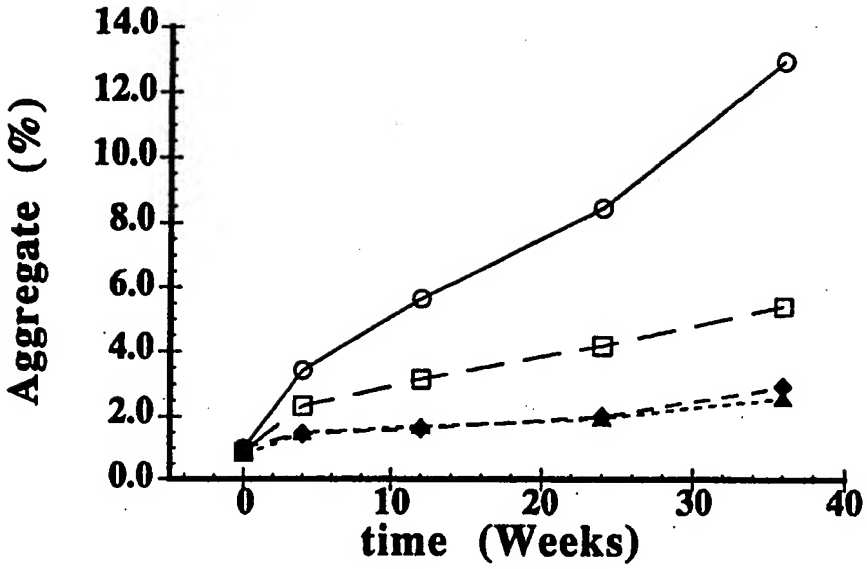

FIG. 19

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 96/12251

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/00 A61K39/395 C07K1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 661 060 A (IMMUNO AG) 5 July 1995 see the whole document ---	1,3,6, 8-12
A	WO 89 09402 A (TORAY INDUSTRIES INC.) 5 October 1989 see the whole document ---	1-3,6, 10,12
A	JOURNAL OF IMMUNOLOGICAL METHODS, vol. 181, no. 1, 12 April 1995, AMSTERDAM, NL, pages 37-43, XP002019425 P. DRABER ET AL.: "Stability of monoclonal IgM antibodies freeze-dried in the presence of trehalose." see abstract --- -/--	1-3,6, 10,12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
25 November 1996	03.12.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Nooij, F

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INTERNATIONAL SEARCH REPORT

Inter onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF IMMUNOASSAY, vol. 16, no. 2, May 1995, NEW YORK, NY, USA, pages 183-197, XP000611616 K. NIELSEN ET AL.: "Stability of freeze-dried horseradish peroxidase-conjugated monoclonal antibodies used in diagnostic serology." see the whole document -----	1-3,6, 10,12

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/12251

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-661060	05-07-95	DE-C-	4344824	31-08-95
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		DE-T-	68920693	24-05-95
		EP-A-	0365685	02-05-90
		US-A-	5262296	16-11-93

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/18, 47/00

A1

(11) International Publication Number: WO 97/17087

(43) International Publication Date: 15 May 1997 (15.05.97)

(21) International Application Number: PCT/US96/16881

(22) International Filing Date: 21 October 1996 (21.10.96)

(30) Priority Data:
08/554,685 7 November 1995 (07.11.95) US

(71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).

(72) Inventors: DE YOUNG, Linda, R.; 301 Shelter Cove, El Granada, CA 94018 (US). LAM, XANTHE, M.; 325 Abbott Avenue, Daly City, CA 94014 (US). NGUYEN, Tue; 1816 Canyon Oak Court, San Mateo, CA 94402 (US). POWELL, Michael, F.; 531 Hugo Street, San Francisco, CA 94122 (US).

(74) Agents: TORCHIA, Timothy, E. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: STABILIZING FORMULATION FOR NGF

(57) Abstract

Formulations are provided comprising NGF and acetate-containing buffer from pH 5 to 6 that provide enhanced stability of NGF for use in promoting nerve cell growth, repair, survival, differentiation, maturation or function.

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STABILIZING FORMULATION FOR NGF

BACKGROUND

Field of the Invention

This invention relates to formulations of nerve growth factor ("NGF") and their use to induce nerve cell growth, differentiation, survival, repair, maturation, or function *in vivo* or *ex vivo*. More particularly, this invention relates to such pharmaceutical compositions having increased stability and solubility characteristics for the NGF component, particularly human recombinant NGF ("rhNGF"), and those making possible the ability to create stable forms thereof for safe, effective therapeutic administration to human subjects.

Description of Related Disclosures

Nerve growth factor (NGF) is a neurotrophic factor required for the growth and survival of sympathetic and sensory neurons during development and in mature animals (1). Clinical indications for recombinant human NGF include peripheral sensory neuropathy and Alzheimer's disease. For example, the systemic administration of NGF has been shown to reduce the sensory neuropathy induced by administration of cisplatin and taxol to mice (2,3). In recent clinical trials, NGF has been administered to humans to improve sensory function in diabetic neuropathies (4).

NGF is currently being developed as a liquid parenteral formulation. The protein stability is complicated beyond the usual chemical and physical degradation pathways due to the dimeric structure of NGF. Protein stability can be further complicated when recombinant protein is a mixture of C-terminally clipped NGF variants. The crystal structure of murine NGF shows 3 antiparallel pairs of b-strands forming a flat surface through which the monomers dimerize (5); the dimer dissociation constant is $\leq 10^{-13}$ M (6, 7). The rearrangement of monomers within dimers, towards an equilibrium dimer distribution, complicates quantification of NGF dimer degradation.

There exists a need for formulations containing NGF that lead to NGF stability while being safe and effective for therapeutic administration to mammals, particularly human subjects.

SUMMARY

The present invention is based on the finding of formulation conditions and methods for stability of NGF in a liquid formulation. It is an object of the present invention to provide a suitable formulation of NGF with enhanced stability of NGF to provide effective induction of nerve cell growth, survival, differentiation, maturation, repair, or function, preferably *in vivo* or *ex vivo*. In various embodiments the formulations can have enhanced stability to agitation, freezing, thawing, light, or storage. It is another object of the invention to provide a stable NGF formulation for use in treating a mammal, preferably human, in need of NGF treatment so as to provide a therapeutically effective amount of NGF. It is further object to provide an NGF formulation with enhanced consistency for improved application to the neuron or mammal. These and other objects will become apparent to those skilled in the art.

The above objects are achieved by providing an NGF formulation comprising an effective amount of NGF in a pharmaceutically acceptable acetate buffer, preferably sodium acetate. In a specific embodiment this formulation contains about 0.1 to 2.0 mg/ml NGF in an acetate buffer from 5 to 50 mM, from pH 5 to 6. The formulation can optionally contain a pharmaceutically acceptable diluent, a pharmaceutically acceptable salt, preferably sodium chloride, or a preservative, preferably benzyl alcohol.

In another embodiment the invention provides a method of producing an NGF formulation produced by the steps including formulating NGF and acetate, and optionally sodium chloride, and further optionally a preservative.

In another embodiment a method is presented by which NGF dimer degradation is quantitated independent of dimer exchange.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the dependence of NGF aggregate formation at 37°C on formulation buffer and pH, quantitated by size-exclusion chromatography, (◇) succinate pH 4.2; (Δ) succinate pH 5.0; (□) succinate pH 5.8; (X) succinate pH 5.0 with 0.05% Tween 20; (▲) acetate pH 5.0; and (■) acetate pH 5.8.

Figure 2 depicts representative RP-HPLC chromatograms for NGF in succinate buffer at pH 5.0 (a) -70°C control and (b) after 38 days of incubation at 37 degrees C.

Figure 3 depicts semilogarithmic plot of the percent NGF monomer remaining after incubation at 37°C for various lengths of time as quantitated by RP-HPLC, (◇) succinate pH 4.2; (Δ) succinate pH 5.0; (□) succinate pH 5.8; (X) succinate pH 5.0 with .05% Tween 20; (▲) acetate pH 5.0; and (■) acetate pH 5.8. Curves are first order fits to the data.

Figure 4 depicts representative IEC chromatograms for NGF in acetate buffer at pH 5.0 after 38 days of incubation at (solid line) -70°C and (dashed line) 37°C. Each dimer appears as a triplet in the chromatogram due to N-terminal Ser to Gly (S1G) conversion (13). The earliest peak in the triplet is the parent dimer, followed by a dimer with a single Ser to Gly conversion, and finally a dimer with a Ser to Gly conversion in both chains.

Figure 5 depicts time dependence of the loss of NGF 118/118 and 117/120 dimers, by IEC, on incubation at 37°C, (Δ) succinate pH 5.0; (□) succinate pH 5.8; (X) succinate pH 5.0 with .05% Tween 20; (▲) acetate pH 5.0; and (■) acetate pH 5.8.

Figure 6 depicts RP-HPLC chromatograms showing the stability of NGF after 1.6 years at (dashed line) 5°C and (solid line) -70°C. The major degradation product at 5°C is Asn93 to iso-Asp93 conversion.

Figures 7A and 7B depict comparisons of NGF (solid line) -70°C control and (dashed line) 5°C IEC chromatograms after 1.6 years of incubation in acetate buffer at pH 5.0, (Figure 7A) no acid treatment, and (Figure 7B) acid treatment of samples prior to analysis.

Figure 8 depicts RP-HPLC chromatograms of 0.1 mg/ml rhNGF in 10 mM acetate at pH 5.5 and 142 mM NaCl stored at 5°C (solid line), 25°C (dashed line), and 40°C (dotted line) for 3 months. Peak (a) contains di-oxidized rhNGF; peak (b) contains deamidated rhNGF; peak (c) contains mono-oxidized rhNGF; peak (d) contains Iso-aspartate; peak (e) contains 120 rhNGF; peak (f) contains 118 rhNGF; peak (g) contains N-

terminally clipped rhNGF; peak (h) contains misfolded rhNGF; and peak (i) contains protein eluted at gradient ramp.

Figure 9 depicts determination of rhNGF monomers (118 and 120) remaining in rhNGF formulations after 12 months at 5 degrees C by reversed-phase HPLC. Formulation A (-⊖-) contains 2mg/ml rhNGF (142mM NaCl, 10 mM acetate, pH 5.5); formulation B (-□-) contains 0.1 mg/mL rhNGF (136mM NaCl, 20 mM acetate, pH 5.5); formulation C (--◊--) contains formulation B plus 0.9% BA; formulation D (--x--) contains formulation B plus 0.25% phenol; formulation E (---+---) contains 0.1 mg/mL rhNGF (136mM NaCl, 20 mM acetate, 0.01%F68, pH 5.5); formulation F (−Δ--) contains formulation E plus 0.9% BA; and formulation G (--■--) contains formulation E plus 0.25% phenol.

Figure 10 depicts determination of rhNGF monomers (118 and 120) remaining in rhNGF formulations after 9 months at 25 degrees C by reversed-phase HPLC. Formulation A (-⊖-) contains 2mg/ml (10 mM acetate, pH 5.5); formulation B (-□-) contains 0.1 mg/ml (20 mM acetate, pH 5.5); formulation C (--◊--) contains formulation B plus 0.9% BA; formulation D (--x--) contains formulation B plus 0.25% phenol; formulation E (--+--) contains 0.1 mg/mL (20 mM acetate, 0.01% F68, pH5.5); formulation F (−Δ--) contains formulation E plus 0.9% BA; and formulation G (--●--) contains formulation E plus 0.25% phenol.

Figure 11 depicts effect of preservative on Iso-aspartate formation of rhNGF in liquid multi-dose formulations stored at 5 degrees C for 12 months as determined by RP-HPLC. Formulation A (-⊖-) contains 2mg/mL (10 mM acetate, pH 5.5); formulation B (-□-) contains 0.1 mg/mL (20 mM acetate, pH 5.5); formulation C (--◊--) contains formulation B plus 0.9% BA; formulation D (--x--) contains formulation B plus 0.25% phenol; formulation E (--+--) contains 0.1 mg/mL (20 mM acetate, 0.01% F68, pH 5.5); formulation F (−Δ--) contains formulation E plus 0.9% BA; and formulation G (--■--) contains formulation E plus 0.25% phenol.

Figure 12 depicts effect of preservative on Iso-aspartate formation of rhNGF in liquid multi-dose formulations stored at 25 degrees C for 9 months as determined by RP-HPLC. Formulation A (-⊖-) contains 2mg/mL (10 mM acetate, pH 5.5); formulation B (-□-) contains 0.1 mg/mL (20 mM acetate, pH 5.5); formulation C (--◊--) contains formulation B plus 0.9% BA; formulation D (--x--) contains formulation B plus 0.25% phenol; formulation E (--+--) contains 0.1 mg/mL (20 mM acetate, 0.01% F68, pH 5.5); formulation F (−Δ--) contains formulation E plus 0.9% BA; and formulation G (--■--) contains formulation E plus 0.25% phenol.

Figure 13 depicts cation exchange HPLC chromatograms of 0.1 mg/ml rhNGF in 10 mM acetate at pH 5.5 and 142 mM NaCl stored at 5 degrees C (solid line), 25 degrees C (dashed line), and 40 degrees C (dotted line) for 3 months. Peak (a) contains mono and di-oxidized 118/118 and oxidized N-terminally clipped rhNGF; peak (b) contains 118/118 rhNGF homodimer; and peak (c) contains Ser-Gly 118/118 rhNGF (1-chain).

Figure 14 depicts determination of rhNGF dimer (118/118) remaining in rhNGF formulations after 12 months at 5 degrees C by cation exchange HPLC. Formulation A (-⊖-) contains 2mg/mL (10 mM acetate, pH 5.5); formulation B (-□-) contains 0.1 mg/mL (20 mM acetate, pH 5.5); formulation C (--◊--) contains formulation B plus 0.9% BA; formulation D (--x--) contains formulation B plus 0.25% phenol; formulation E

(--+--) contains 0.1 mg/mL (20 mM acetate, 0.01% F68, pH 5.5); formulation F (—Δ--) contains formulation E plus 0.9% BA; and formulation G (--●--) contains formulation E plus 0.25% phenol.

Figure 15 depicts determination of rhNGF dimer (118/118) remaining in rhNGF formulations after 9 months at 25 degrees C by cation exchange HPLC. Formulation A (-⊖-) contains 2mg/mL (10 mM acetate, pH 5.5); formulation B (-□-) contains 0.1 mg/mL (20 mM acetate, pH 5.5); formulation C (--◇--) contains formulation B plus 0.9% BA; formulation D (--x--) contains formulation B plus 0.25% phenol; formulation E (--+--) contains 0.1 mg/mL (20 mM acetate, 0.01% F68, pH 5.5); formulation F (—Δ--) contains formulation E plus 0.9% BA; and formulation G (--●--) contains formulation E plus 0.25% phenol.

Figure 16 depicts near UV CD spectrum of rhNGF in 10 mM acetate, 136 mM NaCl, pH 5.5.

Figure 17 depicts a comparison of near-UV CD spectra of rhNGF in the presence (solid line) and absence (dotted line) of 0.9% benzyl alcohol in 20 mM acetate at pH 5.5 and 136 mM NaCl after 24 hours at 25 degrees C.

DETAILED DESCRIPTION

The present invention is based on the discovery that NGF formulated in pharmaceutically acceptable acetate buffer from pH 5 to pH 6 as a pharmaceutical composition has markedly increased stability in these compositions. Acetate concentrations can range from 0.1 to 200 mM, more preferably from 1 to 50 mM, and even more 5 to 30 mM, and most preferably from 10 to 20 mM. One preferred embodiment has 20 mM acetate and another has 10 mM acetate in the administered solution. A preferred acetate salt for enhancing stability and buffering capacity is sodium acetate. However other physiologically acceptable acetate salts can be used, for example potassium acetate. Suitable pH ranges for the preparation of the compositions herein are from 5 to 6, preferably 5.4 to 5.9, more preferably 5.5 to 5.8. A preferred pH is 5.5 which enhances stability and buffering capacity. Another preferred embodiment is pH 5.8.

A "pharmaceutically effective amount" of NGF refers to that amount which provides therapeutic effect in various administration regimens. The compositions herein are prepared containing amounts of NGF from 0.07 to 20 mg/ml, preferably 0.08 to 15 mg/ml, more preferably .09 to 10 mg/ml, and most preferably 0.1 to 2 mg/ml. In a preferred embodiment the NGF concentration is 0.1 mg/ml. In another preferred embodiment the NGF concentration is 2.0 mg/ml. For use of these compositions in administration to human patients suffering from peripheral neuropathies, for example, these compositions may contain from about 0.1 mg/ml to about 2 mg/ml NGF, corresponding to the currently contemplated dosage rate for such treatment. NGF is well-tolerated and higher doses can be administered if necessary as determined by the physician.

Optionally, but preferably, the formulation contains a pharmaceutically acceptable salt, preferably sodium chloride, and preferably at about physiological concentrations. Low concentrations are preferred, e.g., less than about 0.3 M to about .05 M, preferably from 0.16 to 0.20 M NaCl, more preferably 0.13 to 0.15 M. In a preferred embodiment the sodium chloride concentration is 136 mM. In another preferred embodiment the concentration is 142 mM.

Optionally, the formulations of the invention can contain a pharmaceutically acceptable preservative. In some embodiments the preservative concentration ranges from 0.1 to 2.0%, typically v/v. Suitable

preservatives include those known in the pharmaceutical arts. Benzyl alcohol, phenol, m-cresol, methylparaben, and propylparaben are preferred preservatives. Benzyl alcohol is a particularly preferred preservative that results in enhanced NGF stability. A particularly preferred benzyl alcohol concentration is 0.7 to 1.2%, more preferably 0.8 to 1.0%, with a particularly preferred concentration of 0.9%.

Optionally, the formulations of the invention can include a pharmaceutically acceptable surfactant. Preferred surfactants are non-ionic detergents. Preferred surfactants include Tween 20 and pluronic acid (F68). F68 is particularly preferred for enhancing NGF stability. Suitable surfactant concentrations are 0.005 to 0.02%. A preferred concentration for surfactant is 0.01%. Surfactants are used to minimize particulate formation.

In a particularly preferred embodiment the composition contains an NGF concentration of 0.1 mg/ml, a sodium acetate concentration of 20 mM, pH 5.5, a sodium chloride concentration of 136 mM, and benzyl alcohol concentration at 0.9% (v/v). In another embodiment the NGF concentration is 2.0 mg/ml, the sodium acetate concentration is 10 mM, pH 5.5, and the sodium chloride concentration is 142 mM.

In another embodiment of the invention is provided a kit for NGF administration, which includes a vial or receptacle containing a pharmaceutical composition of the invention comprising a pharmaceutically effective amount of nerve growth factor and a pharmaceutically acceptable acetate-containing buffer. A preferred vial volume is one suitable for multi-dose use--allowing repeated withdrawal of sample. The increased stability attained with the formulations of the invention allow multi-dose liquid formulation. Typically a multi-dose vial will provide sufficient formulation to supply sufficient dosage for one patient for one month, preferably one week. For example, the composition volume generally ranges from 0.3 to 10.0 ml and more preferably from 1.6 to 2.0 ml, depending on dose concentration, frequency and ease of use. For example, a volume of 1.8 ml is convenient when either 0.3 ug/kg or 0.1 ug/kg are used, allowing 7 or 24 doses, respectively. When a light sensitive component, such as benzyl alcohol is present, the vial is protected from intense light. Generally it is sufficient to store the vial in a darkened refrigerator or within an opaque box. However, the vial walls can comprise light transmission reducing materials. For example, translucent amber or brown vials or an opaque vail can be used. In preferred embodiments the vial contains multi-dose formulation. For a vial configuration, a selected multi-dose liquid formulation can be filled in 3 cc Type I glass vial with 1.8 mL fill volume. Selection of stopper will be based on compatibility of different types of stopper with the selected formulation.

Compositions of the invention are typically stored at 2 to 8 degrees C. The formulations are stable to numerous freeze thaw cycles as shown herein.

In another embodiment the formulation is prepared with the above acetate concentrations.

A preferred means of preparing a formulation is to dialyze a bulk NGF solution into the final formulation buffer. Final NGF concentrations are achieved by appropriate adjustment of the formulation with formulation buffer absent NGF. Also provided are methods for the preparation of the composition of claim 1 comprising the steps of compounding said NGF and acetate-containing buffer. Also provided are methods of increasing the stability of NGF in a pharmaceutical composition containing NGF as active principle, comprising incorporating acetate in said composition, wherein said acetate is present in an amount and pH effective to increase the stability of the NGF.

The compositions hereof including lyophilized forms, are prepared in general by compounding the components using generally available pharmaceutical compounding techniques, known per se. Likewise, standard lyophilization procedures and equipment well-known in the art are employed. A particular method for preparing a pharmaceutical composition of NGF hereof comprises employing purified (according to any standard protein purification scheme) NGF, preferably rhNGF, in any one of several known buffer exchange methods, such as gel filtration or dialysis.

Nerve growth factor ("NGF") is a 120 amino acid polypeptide homodimeric protein that has prominent effects on developing sensory and sympathetic neurons of the peripheral nervous system. NGF acts via specific cell surface receptors on responsive neurons to support neuronal survival, promote neurite outgrowth, and enhance neurochemical differentiation. NGF actions are accompanied by alterations in neuronal membranes, in the state of phosphorylation of neuronal proteins, and in the abundance of certain mRNAs and proteins likely to play a role in neuronal differentiation and function. (Connolly *et al.*, J. Cell. Biol. 90:176-180 [1981]; Skaper and Varon, Brain Res. 197:379-389 [1980]; Yu, *et al.*, J. Biol. Chem. 255:10481-10492 [1980]; Haleqoua and Patrick, Cell 22:571-581 [1980]; Tiercy and Shooter, J. Cell. Biol. 103:2367-2378 [1986]).

Forebrain cholinergic neurons also respond to NGF and may require NGF for trophic support. (Hefti, J. Neurosci., 6: 2155 [1986]). Indeed, the distribution and ontogenesis of NGF and its receptor in the central nervous system (CNS) suggest that NGF acts as a target-derived neurotrophic factor for basal forebrain cholinergic neurons (Korsching, TINS, pp 570-573 [Nov/Dec 1986]).

Little is known about the NGF amino acid residues necessary for the interaction with the trkA-tyrosine kinase receptor. Significant losses of biological activity and receptor binding were observed with purified homodimers of human and mouse NGF, representing homogenous truncated forms modified at the amino and carboxy termini. The 109 amino acid species (10-118)hNGF, resulting from the loss of the first 9 residues of the N-terminus and the last two residues from the C-terminus of purified recombinant human NGF, is 300-fold less efficient in displacing mouse [^{125}I]NGF from the human trkA receptor compared to (1-118)hNGF. It is 50- to 100-fold less active in dorsal root ganglion and sympathetic ganglion survival compared to (1-118)hNGF. The (1-118)hNGF has considerably lower trkA tyrosine kinase autophosphorylation activity. A preferred form is the 118 amino acid human NGF, which is more preferable as a homodimer.

The formulations of the invention include the pantropic neurotrophin pantropic NGF. Pantropic NGF is a pantropic neurotrophin which has an amino acid sequence homologous to the amino acid sequence of NGF, with domains which confer other neurotrophin specificities. In the preferred embodiment, the domains are substituted for NGF residues; that is, some number of amino acids are deleted from the NGF sequence, and an identical or similar number of amino acids are substituted, conferring an additional specificity. For example, a pantropic NGF is made with a D16A substitution, which confers BDNF specificity. Optionally, substitutions in the pre-variable region 1 (V18E+V20L+G23T) and in variable region 4 (Y79Q+T81K+H84Q+F86Y+K88R) are included. Alternatively, the substitutions in the pre-variable region 1 can be made with only single amino acid substitutions in variable region 4; for example, V18E+V20L+G23T and one of Y79Q, T81K, H84Q, F86Y, or K88R may be made.

The chemical and physical stability of recombinant human nerve growth factor (NGF) in aqueous solution was investigated between 5 and 37°C, in the pH range 4.2 to 5.8. NGF chemical stability increased with increasing pH. In succinate buffer at pH 5.8, NGF physical stability decreased due to protein aggregation. Based on both the 5°C stability data and accelerated degradation studies at 37°C, the optimal formulation was found to be acetate buffer at pH 5.8. Reversed-phase HPLC was the primary stability indicating method, showing conversion of Asn-93 to iso-Asp to be the primary degradation pathway at 5°C. Quantitation of NGF degradation by cation exchange chromatography was complicated by the rearrangement of the NGF monomer variants into various mixed dimers over time (dimer exchange). Treatment of samples and controls with dilute acid rapidly equilibrated the monomer distribution in the dimers, allowing NGF degradation to be quantitated in the absence of dimer exchange.

Benzyl alcohol and phenol were evaluated for their compatibility and stability with rhNGF in two liquid formulations for multi-use purposes. These two formulations consist of 0.1 mg/mL protein in 20 mM sodium acetate at pH 5.5 and 136 mM sodium chloride with and without 0.01% pluronic acid (F68) as surfactant. The final concentrations of benzyl alcohol and phenol in each of these two formulations were 0.9 and 0.25%, respectively. Based on the 12 month stability data, rhNGF is more stable with benzyl alcohol than phenol in these formulations. Benzyl alcohol preserved rhNGF formulation with the presence of surfactant is as stable as the formulation with no surfactant added, indicating that the addition of F68 to rhNGF multi-dose formulation is not required for stability purpose. Therefore, a formulation consisting of 0.1 mg/mL protein in 20 mM acetate, 136 mM NaCl, 0.9% benzyl alcohol, pH 5.5 is recommended for rhNGF used for multiple dosing in Phase III clinical trails. This rhNGF multi-dose formulation passed the USP and EP preservative efficacy test after 6 months at 5 degrees C, and is as stable as the current liquid formulation at 2 mg/mL. However, the formulation should avoid exposure to intensive light due to the presence of benzyl alcohol as preservative which is light sensitive.

In general, the compositions may contain other components in amounts preferably not detracting from the preparation of stable, liquid or lyophilizable forms and in amounts suitable for effective, safe pharmaceutical administration.

In order that materials like NGF be provided to health care personnel and patients, these materials must be prepared as pharmaceutical compositions. Such compositions must be stable for appropriate periods of time, must be acceptable in their own right for administration to humans, and must be readily manufacturable. An example of such a composition would be a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the medicinal agent contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of suitable pharmaceutically acceptable diluent(s), such as sterile water for injection or sterile physiological saline solution, and the like.

NGF formulations of the invention are believed to be useful in promoting the development, maintenance, or regeneration of neurons *in vivo*, including central (brain and spinal chord), peripheral (sympathetic, parasympathetic, sensory, and enteric neurons), and motorneurons. Accordingly, NGF formulations of the invention are utilized in methods for the treatment of a variety of neurologic diseases and disorders. In a preferred embodiment, the formulations of the present invention are administered to a patient to treat neural disorders. By "neural disorders" herein is meant disorders of the central and/or peripheral nervous system that are associated with neuron degeneration or damage. Specific examples of neural disorders include, but are not limited to, Alzheimer's disease, Parkinson's disease, Huntington's chorea, stroke, ALS, peripheral neuropathies, and other conditions characterized by necrosis or loss of neurons, whether central, peripheral, or motorneurons, in addition to treating damaged nerves due to trauma, burns, kidney disfunction, injury, and the toxic effects of chemotherapeutics used to treat cancer and AIDS. For example, peripheral neuropathies associated with certain conditions, such as neuropathies associated with diabetes, AIDS, or chemotherapy may be treated using the formulations of the present invention. It also is useful as a component of culture media for use in culturing nerve cells *in vitro* or *ex vivo*.

In various embodiments of the invention, NGF formulations are administered to patients in whom the nervous system has been damaged by trauma, surgery, stroke, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, or toxic agents, to promote the survival or growth of neurons, or in whatever conditions have been found treatable with NGF. For example, NGF formulation of the invention can be used to promote the survival or growth of motorneurons that are damaged by trauma or surgery. Also, NGF formulations of the invention can be used to treat motoneuron disorders, such as amyotrophic lateral sclerosis (Lou Gehrig's disease), Bell's palsy, and various conditions involving spinal muscular atrophy, or paralysis. NGF formulations of the invention can be used to treat human neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, Huntington's chorea, Down's Syndrome, nerve deafness, and Meniere's disease. NGF formulations of the invention can be used as cognitive enhancer, to enhance learning particularly in dementias or trauma. Alzheimer's disease, which has been identified by the National Institutes of Aging as accounting for more than 50% of dementia in the elderly, is also the fourth or fifth leading cause of death in Americans over 65 years of age. Four million Americans, 40% of Americans over age 85 (the fastest growing segment of the U.S. population), have Alzheimer's disease. Twenty-five percent of all patients with Parkinson's disease also suffer from Alzheimer's disease-like dementia. And in about 15% of patients with dementia, Alzheimer's disease and multi-infarct dementia coexist. The third most common cause of dementia, after Alzheimer's disease and vascular dementia, is cognitive impairment due to organic brain disease related directly to alcoholism, which occurs in about 10% of alcoholics. However, the most consistent abnormality for Alzheimer's disease, as well as for vascular dementia and cognitive impairment due to organic brain disease related to alcoholism, is the degeneration of the cholinergic system arising from the basal forebrain (BF) to both the codex and hippocampus (Bigl *et al.* in Brain Cholinergic Systems, M. Steriade and D. Biesold, eds., Oxford University Press, Oxford, pp.364-386 (1990)). And there are a number of other neurotransmitter systems affected by Alzheimer's disease (Davies *Med. Res. Rev.* 3:221 (1983)). However, cognitive impairment, related for example to degeneration of the cholinergic neurotransmitter system, is not limited to individuals suffering

from dementia. It has also been seen in otherwise healthy aged adults and rats. Studies that compare the degree of learning impairment with the degree of reduced cortical cerebral blood flow in aged rats show a good correlation (Berman *et al. Neurobiol. Aging* 9:691 (1988)). In chronic alcoholism the resultant organic brain disease, like Alzheimer's disease and normal aging, is also characterized by diffuse reductions in cortical cerebral blood flow in those brain regions where cholinergic neurons arise (basal forebrain) and to which they project (cerebral cortex) (Lofti *et al., Cerebrovasc. and Brain Metab. Rev* 1:2 (1989)). Such dementias can be treated by administration of NGF formulations of the invention.

Further, NGF formulations of the invention are preferably used to treat neuropathy, and especially peripheral neuropathy. "Peripheral neuropathy" refers to a disorder affecting the peripheral nervous system, most often manifested as one or a combination of motor, sensory, sensorimotor, or autonomic neural dysfunction. The wide variety of morphologies exhibited by peripheral neuropathies can each be attributed uniquely to an equally wide number of causes. For example, peripheral neuropathies can be genetically acquired, can result from a systemic disease, or can be induced by a toxic agent. Examples include but are not limited to diabetic peripheral neuropathy, distal sensorimotor neuropathy, or autonomic neuropathies such as reduced motility of the gastrointestinal tract or atony of the urinary bladder. Examples of neuropathies associated with systemic disease include post-polio syndrome; examples of hereditary neuropathies include Charcot-Marie-Tooth disease, Refsum's disease, Abetalipoproteinemia, Tangier disease, Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, and Dejerine-Sottas syndrome; and examples of neuropathies caused by a toxic agent include those caused by treatment with a chemotherapeutic agent such as vincristine, cisplatin, methotrexate, or 3'-azido-3'-deoxythymidine.

A therapeutically effective dose of an NGF formulation is administered to a patient. By "therapeutically effective dose" herein is meant a dose that produces the effects for which it is administered. The exact dose will depend on the disorder to be treated, and will be ascertainable by one skilled in the art using known techniques. In general, the NGF formulations of the present invention are administered at about 0.01 µg/kg to about 100 mg/kg per day. Preferably, from 0.1 to 0.3 ug/kg. In addition, as is known in the art, adjustments for age as well as the body weight, general health, sex, diet, time of administration, drug interaction and the severity of the disease may be necessary, and will be ascertainable with routine experimentation by those skilled in the art. Typically, the clinician will administer NGF formulations of the invention until a dosage is reached that repairs, maintains, and, optimally, reestablishes neuron function. The progress of this therapy is easily monitored by conventional assays.

A "patient" for the purposes of the present invention includes both humans and other mammals. Thus the methods are applicable to both human therapy and veterinary applications.

Therapeutic formulations of NGF are prepared by mixing NGF having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences). Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and will not significantly decrease NGF stability in the formulations as taught herein. Such compounds include antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as

polyvinylpyrrolidone, amino acids such as histidine, methionine, glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or non-ionic surfactants such as Tween, Pluronics or PEG.

NGF formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes. Ordinarily NGF formulations of the present invention will be stored in liquid form at 2 to 8 degrees C. The formulations are suitable for frozen storage with repeated cycles of thawing and freezing.

Therapeutic NGF compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

NGF optionally is combined with or administered in concert with other neurotrophic factors including NT-4/5, NT-3, and/or BDNF and is used with other conventional therapies for nerve disorders.

The administration of the formulations of the present invention can be done in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, or intraocularly. The formulations can be administered continuously by infusion into the fluid reservoirs of the CNS, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances, for example, in the treatment of wounds, the formulations may be directly applied as a solution or spray.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLES

Example I

Materials

Recombinant human nerve growth factor (NGF) was produced in Chinese hamster ovary cells and purified by reversed-phase (RP-HPLC) and ion-exchange chromatography (IEC) as described previously (8). HPLC grade acetonitrile, and TFA were used for RP-HPLC. All other chemicals were USP grade. Sterile type I, clear glass, 2 cc vials were purchased from Wheaton and used with siliconized, Teflon-coated, butyl rubber stoppers.

Methods

NGF was dialyzed into 10 mM sodium acetate, 142 mM sodium chloride, at pH 5.0 and 5.8, and into 10 mM sodium succinate, 142 mM NaCl, at pH 4.2, 5.0, and 5.8, and adjusted to 10 mg/ml. Tween 20 was also added to a succinate pH 5.0 formulation to determine if surfactant would reduce NGF aggregation (10 mM sodium succinate, 142 mM NaCl, 0.05% Tween 20).

Vials were aseptically filled with 0.3 ml of NGF formulation and stored at 5, 25, and 37°C (25°C data not reported here). Controls were stored at -70°C where no significant degradation has been observed. At each time point, 50 µl aliquots were removed from individual vials and stored at -70°C until analysis.

HPLC Analysis. Cation exchange HPLC (IEC) was carried out on a HP 1090 system using a Tosohas sulpho-propyl TSK-SP-5PW (7.5 x 75 mm) column with 10 m particles. Mobile phases were (A) 10 mM sodium phosphate, 5% (v/v) acetonitrile, pH 7.0 and (B) A + 1.0 M ammonium chloride. NGF was eluted at 35°C (0.5 ml/min) with a linear gradient of 20-40% B from 5 to 60 minutes. The control and 1.6 year samples at 5°C were also assayed after "acid-treatment" to bring the distribution of monomer variants in the dimers to equilibrium (8, 9). These samples were adjusted to pH 3.5 with HCl and incubated at 37°C for 2 hours (results at 2 and 4 hours were equivalent). A YMC C4, 5 µm (4.6 x 250 mm) column was used for reversed-phase HPLC (RP-HPLC) on a HP 1090 system at 25°C. NGF was eluted (0.5 ml/min) using a linear gradient of 26-30% B in A (B = 0.05% TFA in acetonitrile and A = 0.05% TFA in water) run between 5 and 40 minutes. Size exclusion HPLC ("SEC-HPLC") was carried out using a Perkin Elmer Series 410 Bio LC Pump with a Perkin Elmer LC 90 Spectrophotometric UV Detector and a Tosohas TSK 2000 SWXL, 5 µm (7.8 x 300 mm) column. This SEC column was run at 0.5 ml/min using a 0.2 M potassium phosphate, 0.45 M potassium chloride mobile phase, at pH 7.0. For SEC UV detection was at 280 nm; for RP-HPLC and IEC, at 214 nm. For all assays 50 mg of NGF were injected.

SDS-PAGE. Samples were diluted into Novex tricine SDS sample buffer and incubated for 1 hour at 50°C. Non-reduced SDS-PAGE was run on Novex tricine gels containing 10% acrylamide followed by Coomassie Blue staining. Molecular weights were estimated using Bio-Rad low molecular weight markers.

Neurite Outgrowth Assay. The biological activity of NGF was determined using the PC12 assay developed by Greene (10) and modified as described by Schmelzer et al (8).

Hemolysis. All formulations were tested for hemolytic activity. The hemolysis procedure was that of Reed and Yalkowsky (11) except that equal volumes of washed human red blood cells and formulation were incubated at 37°C for 30 minutes before analysis.

Results

Formulation development of NGF requires condition be found for which the protein shows ≥ 1.5 years of chemical and physical stability at 2-8°C. We determined the approximate pH of maximal NGF stability by ascertaining NGF stability in succinate buffer at pH 4.2, 5.0, and 5.8, and acetate buffer at pH 5.0 and 5.8. NGF stability decreases above pH 6.0. The assays used to measure protein stability were IEC, SEC, RP-HPLC, SDS-PAGE, and the PC12 bioactivity assay. Formulation biocompatibility was determined by hemolysis testing. Stability of NGF at 37°C.

Aggregation of NGF. The dimer/monomer equilibrium constant for murine NGF is smaller than 10-13 M at pH 4-7 (6, 7, 9, 12). NGF, therefore, assayed primarily as a dimer in the neutral pH SEC assay. A small amount of aggregated NGF (tetramer based on molecular weight standards) was observed in the control sample. This tetramer peak area increased with time at 37°C. A leading shoulder on this peak, indicating larger aggregates, was observed for all formulations after 38 days at 37°C. The time dependencies of aggregate formation for the various formulations are shown in Figure 1. The succinate pH 5.8 formulation had the greatest aggregation rate. All other formulations had similar rates of aggregate formation. The addition of the surfactant Tween 20 offered no protection against aggregation in the pH 5.0 succinate formulation. During preparation,

the NGF pH 5.8 succinate formulation had to be filtered through a 47 mm diameter 0.22 mm filter, whereas all other formulation were filterable through a 25 mm diameter filter. This is consistent with the high rate of aggregation observed at 37°C in succinate buffer at pH 5.8.

Aggregation was also monitored using non-reduced SDS-PAGE (gels not shown). In the -70°C control samples 3 bands were observed: monomer at 13.5 kDa, a very faint dimer band at approximately 26 kDa, and a slightly more intense band at 31 kDa. The 26 and 31 kDa bands became more intense on incubation at elevated temperatures. A small amount of large molecular weight aggregate (> 97 kDa) was observed in all formulations after 38 days at 37°C. The intensity of this band was greatest in the pH 5.8 succinate formulation, consistent with the poor filterability and high aggregation rate observed by SEC for this formulation. Tween 20 prevented the formation of this high molecular weight aggregate at pH 5.0. With the exception of succinate at pH 5.8, these sizing methods do not differentiate between the quality of the NGF formulations.

NGF Monomer and Degradation Product Quantitation. The NGF used in these studies consisted of a 1:9:1 ratio of the three monomeric polypeptides containing 120, 118, and 117 amino acids. The 118 amino acid variant was produced by clipping of Ala120 and Arg119 from the C-terminus of the 120 parent; the 117 variant had an additional clip, Arg118 (8). At pH 5.0, the 117 variant has two fewer positive charges, and the 118 variant one fewer positive charge than the 120 parent. There is no significant difference in the bioactivity of the homodimers and heterodimers formed by the 117, 118, and 120 variants as measured by the PC12 and chick dorsal root ganglion assays (8). In the acidic, organic, RP mobile phase where NGF dissociates to monomer (8), the elution order is 120 before 118, then 117. Typical RP-HPLC chromatograms for NGF stored in pH 5.0 succinate buffer, for 38 days, at -70°C and 37°C are shown in Figure 2. At elevated temperature, peak area is lost from the peaks defined as NGF (the sum of the 118 and 120 monomer peaks) with the iso-Asp, oxidized, and other NGF degradation peaks increasing in area. The 117 peak area was not included in the definition of NGF due to coelution of degradation products with this peak at elevated temperatures. The time dependence of NGF degradation at 37°C, and the apparent first order rate constants for this degradation, are shown in Figure 3 and Table 1, respectively.

Table 1. Apparent First-Order Rate Constants for NGF Degradation at 37°C as Determined by RP-HPLC.

Buffer	pH	k (day-1)
Succinate	4.2	2.2 x 10-2 ± 1.0 x 10-3
	5.0	1.1 x 10-2 ± 6.3 x 10-4
(+Tween 20)	5.0	1.1 x 10-2 ± 7.1 x 10-4
	5.8	5.7 x 10-3 ± 9.7 x 10-4
Acetate	5.0	7.9 x 10-3 ± 8.0 x 10-4
	5.8	4.0 x 10-3 ± 2.9 x 10-4

NGF stability decreased as the pH was lowered. In both the acetate and succinate pH 5.8 buffers NGF stability was greater than at pH 5.0. In succinate buffer at pH 4.2, the NGF degradation rate is further increased, with several hydrophobic degradation products being observed, possibly due to acid-induced

cleavage at the Asp60-Pro61 linkage. Tween 20 had no affect on NGF stability in succinate buffer at pH 5.0 (Figure 3). The acetate formulation appears to be somewhat better in maintaining NGF stability.

NGF Dimer Distribution. The three NGF monomers containing 117, 118, and 120 amino acids may combine to form the 117/117, 118/118 and 120/120 homodimers and the 117/118, 118/120, and 117/120 heterodimers. Association of these NGF variants has been shown to be random, with no monomer appearing to prefer any other (8, 9). The dynamic dissociation and reassociation of monomers to form various dimers (dimer exchange) is accelerated by low pH and increased temperature (9). For a random association process at equilibrium, and an initial 117/118/120 ratio of 1:9:1, the 118/118 homodimer will be the dominant dimer species with smaller amounts of the 117/118 and 118/120 dimers being formed.

The 118/118 and 117/120 dimers have the same effective net charge in the chosen IEC mobile phase and therefore coelute on IEC during NGF purification. This results in an initial non-equilibrium distribution of the monomer variants in NGF dimers in the NGF product. The 117/120 and 118/118 dimers dissociate giving the 117 and 120 monomers which will reassociate most frequently with 118 monomer to form 117/118 and 118/120 dimers. Due to the different charges on the monomers, the expected elution order of these dimers on cation-exchange chromatography is:

117/117 < 117/118 < 118/118 = 117/120 < 118/120 < 120/120.

The most populated dimers are distinguishable by IEC (8) as shown in Figure 4.

Representative IEC chromatograms for NGF at pH 5.0 in succinate buffer after 38 days at -70°C and 37°C are shown in Figure 4. During NGF production, a fraction of the N-terminal serine residues are converted to glycine with no affect on NGF activity (13). NGF is quantitated here as the sum of the 118/118 homodimer and the 118/118 dimer with a Ser1 to Gly1 conversion in one of the two monomers (13) (and any coeluting 117/120 variants); the 117/118 and 118/120 peak areas are not included due to degradation products coeluting with these peaks. The rate of loss of NGF, as monitored by IEC at 37°C, is shown in Figure 5. The degradation kinetics for the 118 dimer are multiphasic. The loss in main peak area before 13 days is largely due to rearrangement of the monomer variants between the possible dimer types. The data after 13 days more accurately describes NGF chemical degradation. NGF is most stable in the acetate formulations at pH 5.0 and 5.8, which have similar stability. NGF in succinate buffer at pH 5.8 and pH 5.0, with and without 0.05% Tween 20, all have similar stabilities. The hemolytic activity of each of the NGF formulations was also tested. None of the formulations showed significant red blood cell hemolysis (<0.1%). The bioactivity of NGF in each of the formulations was also determined, using the neurite extension PC12 assay. NGF was bioactive in all of the formulations after 38 days at 37°C. The large assay variability (approximately 50% error) did not allow quantitative bioactivity differences between these formulations to be determined.

A liquid formulation for NGF preferably has an adequate shelf-life at 5°C. The accelerated stability data at 37°C showed NGF to be most stable in acetate buffer. Based on this data, NGF stability in the acetate pH 5.0 and 5.8 formulations was investigated for 1.6 years at 5°C. RP-HPLC chromatograms at pH 5.0 for the 1.6 year -70°C control and 5°C samples are shown in Figure 6. The major degradation product was Asp-93 conversion to iso-Asp; smaller amounts of Met-37 and Met-92 oxidation were

observed. The apparent first order rate constants for NGF degradation, quantitated by RP-HPLC, are 1.4 x 10-4 ± 1.7 x 10-5 d -1 and 6.8 x 10-5 ± 7.0 x 10-6 d-1 at pH 5.0 and 5.8, respectively. At 5°C, IEC shows that NGF stability is approximately the same at pH 5.0 and 5.8, consistent with the 37°C IEC data. Aggregation of the NGF dimers was not a significant degradation pathway at 5°C, only a 1% increase in aggregate was observed over 1.6 years of storage at 5°C.

The interpretation of the IEC data at both 5°C and 37°C, is complicated by dimer exchange, the exchange rate being slower at the lower temperature. To improve IEC quantitation, the dimer distribution was brought to equilibrium by incubation at pH 3.5 for 2 hours at 37°C prior to IEC analysis (8,9,14). No new degradation products were observed after this treatment. The acetate pH 5.8 samples after 1.6 years of incubation at 5°C are compared with controls before and after "acid treatment" in Figure 7. The loss of main peak area to the peripheral peaks due to dimer exchange was eliminated by acid treatment, revealing the true degradation of NGF. Quantitation after acid treatment showed that 94 and 92% of the NGF main peaks remain after 1.6 years at 5°C at pH 5.0 and pH 5.8, respectively, compared to 84 and 87% without acid treatment. For comparison, RP-HPLC analysis showed 93 and 96% of the NGF 118 and 120 monomers remaining at pH 5.0 and pH 5.8, respectively.

NGF chemical stability was shown to increase with pH, the pH of maximal stability being near pH 5.8. At a fixed pH, the RP-HPLC and IEC data at 5 and 37°C were consistent in showing NGF chemical stability to be greater in acetate than succinate buffer. In addition, NGF aggregation was not a significant degradation pathway, except at pH 5.8 in succinate buffer. A complicating factor in the determination of NGF stability is that dimer exchange contributes to the apparent degradation of NGF dimers as determined by IEC. A more accurate representation of NGF chemical degradation can be obtained by pretreating the controls and samples with acid at 37°C to bring the dimer distribution to equilibrium. Taken together, these data show that the optimal formulation and storage conditions for NGF stability are acetate butter at pH 5.8 with storage at 5°C.

Example II

Results from Phase II clinical trials indicate that patients with peripheral neuropathy disease require three dosings per week of rhNGF at either 0.3 or 0.1 μg/kg. This means that only 21 or 7 μg per dosing of rhNGF is needed for an average patient of body weight 70 kg. Using the current rhNGF liquid formulation (2 mg/mL in 10 mM sodium acetate, pH 5.5, 142 mM NaCl) and vial configuration (0.7 mL per vial) would have wasted a lot of drug product. Therefore, a new rhNGF formulation at low concentration, preferably multi-dose configuration, is required to reduce the cost and wastage of the product. The purpose of this study was to develop a stable multi-dose liquid formulation for rhNGF at 0.1 mg/mL with 1.8 mL fill in 3 cc glass vial for use in Phase III clinical trails. With this new configuration, each vial will give 180 μg protein and will provide at least 7 doses at the high dosing level (0.3 μg/kg) and 24 doses at the low dosing level (0.1 μg/mL).

In this study, the results on compatibility and stability of preservative containing 0.1 mg/ml rhNGF multi-dose liquid formulations at pH 5.5 are presented. A comparison between the stability of the new multi-dose liquid formulations at 0.1 mg/mL rhNGF and the current 2 mg/mL rhNGF formulation was also studied.

Results on agitation, freezing and thawing, and light compatibility studies of the lead multi-dose liquid formulations for 0.1 mg/mL rhNGF were also reported.

In this study, rhNGF concentrated bulk formulated at 11.6 mg/mL in 10 mM sodium acetate, 142 mM sodium chloride at pH 5.5 with 20 mL filled in 100 cc glass vials was used. All chemical reagents and materials used in this Example are listed in Table 2.

Table 2: List of Materials

-rhNGF concentrated bulk, 11.6 mg/mL, in 10 mM sodium acetate, 142 mM sodium chloride, pH 5.5
-Sodium acetate trihydrate, Genentech Release Materials Code G20136, Lot #S0766
-Glacial acetic acid, Release Materials Code G20027-01, Lot S0567
-Sodium Chloride, Release Materials Code G20136, Lot S1152
-Benzyl alcohol, Release Materials Code G20226, Lot C0200
-m-cresol, Sigma, Lot 107F-3497
-Methylparaben, Napp Chemical Inc., Lot LM 86-6285
-Propylparaben, Napp Chemical Inc., LL86-6241
-Phenol, Release Materials Code G20136, Lot 620015, Lot B0901
-Polysorbate 20, Release Materials Code G20091, Lot A1408
-Pluronic acid (F68), Release Materials Code GXXXX, Lot XXXX
-Sterile, pryogen-free non-siliconized Type I clear glass 3 cc vials (Wheaton Tubing Products); prepared in Phase V per standard procedures
-Sterile 13 mm Purcoat rubber stoppers, Clinical manufacturing, Genentech, Inc.
-13 mm aluminum flip-off cap, Clinical manufacturing, Genentech, Inc.

Methods

rhNGF Multi-dose Liquid Formulations Preparation. rhNGF concentrated bulk was dialyzed into a formulation buffer consisting of 20 mM sodium acetate, 136 mM sodium chloride at pH 5.5 by ultrafiltration using Amicon Centriprep™ concentrator with molecular weight cutoff of 10,000 KD. This reformulated rhNGF bulk was then diluted to 0.15 mg/mL using the same formulation buffer for dialysis. Preservatives and surfactants used for compatibility screening and formulation development studies were added to this diluted rhNGF solution at their tested concentrations. Protein concentration for each formulation was then adjusted to 0.1 mg/mL by UV analysis using the appropriate formulation buffer. A list of preservatives and their concentrations used for physical compatibility with rhNGF in liquid formulations are given in Table 3.

Table 3: List of Preservative Screening Formulations for 0.1 mg/mL rhNGF

Formulation buffer	Surfactant	Preservative
20 mM acetate, pH 5.5 136 mM NaCl	none	0.9% benzyl alcohol 0.25% phenol 0.45% phenol 0.25% m-cresol 0.18% methylparaben 0.02% propylparaben
20 mM acetate, pH 5.5 136 mM NaCl	0.01% Tween 20	0.9% benzyl alcohol 0.25% phenol 0.45% phenol 0.25% m-cresol 0.18% methylparaben 0.02% propylparaben
20 mM acetate, pH 5.5 136 mM NaCl	0.01% F68	0.9% benzyl alcohol 0.25% phenol 0.45% phenol 0.25% m-cresol 0.18% methylparaben 0.02% propylparaben

Experimental Design

All rhNGF multi-dose liquid formulations prepared were sterile filtered through 0.22 μm filter prior to filling. Each formulations were aseptically filled into Type I, clear glass, 3 cc Wheaton vials with a fill volume of 1.8 mL. Vials were stoppered with 13 mm Purcoat stoppers and hand crimped with 13 mm aluminum flip-off caps.

For the preservative screening study, samples were stored at room temperature for 24 hours to determine physical compatibility. For the formulation development study, samples were stored at -70, 5, 25 and 40°C. At each timepoint, one sample/formulation/temperature was assayed.

Agitation studies were carried out at room temperature on the current 2 mg/mL rhNGF formulation, the multi-dose formulations that contain either 0.9% benzyl alcohol or 0.25% phenol in the absence of surfactant, and the 0.1 mg/mL rhNGF control that contains no surfactant and preservative. A 3 cc vial of each formulation tested was secured to a laboratory bench top shaker (Glas-Col) and agitated at 80 rpm for 6 and 24 hours. Samples collected after 6 and 24 hours of shaking were assayed by SE-HPLC, RP-HPLC, ELISA and RRA.

Freezing and thawing cycling was performed on the same formulations that used for agitation studies. One vial from each formulation tested was placed in -70°C freezer and allowed to freeze for 24 hours. After 24 hours of freezing, samples were thawed at 5°C for 24 hours. This freezing and thawing procedure was repeated up to 3 times. Samples collected at the end of the third cycle were assayed by SE-HPLC, RP-HPLC, ELISA and RRA.

The effect of light on stability of rhNGF was studied on the same formulations that used for agitation studies. One vial from each formulation was placed in a light box (Forma Scientific, Model 3890) under high

intensity fluorescent light for 5 weeks. Control vials wrapped with aluminum foil were also placed in the light box. Light intensity was 20,000 lux which was about 15-20 times that of indoor fluorescent light, and the temperature of the light box was maintained at 28°C. Samples were assayed at 2 and 5 weeks by SEC-HPLC, ELISA and RRA.

Analytical Methodology

A. UV Analysis. rhNGF concentration was determined by scanning from 240 to 360 nm using an HP 8452A UV-Vis spectrophotometer. Formulation buffer was used as a reference to blank the instrument, and the protein concentration in mg/mL was calculated from (A280-320)/1.5, where 1.5 is the extinction coefficient of rhNGF in mL/(mg.cm).

B. HPLC Analysis. The following HPLC methods were used.

Reversed-Phase HPLC

column:	YMC C4, 5 µm, 4.6 x 250 mm
mobile phase:	A: 0.05% (v/v) TFA, water
	B: 0.05% (v/v) TFA, 100% AcCN
gradient:	25-27% B (26'), 27-50% B (4'), 50-80% B (1'),
	80-25% B (4'), 25% B (20')
flow rate:	1 mL/min
run time:	55 min
temp:	25°C
LC:	HP-1090
detection:	214, 280 nm
injection:	15 µg

Size Exclusion HPLC

column:	Tosohaas TSK 2000SWXL, 5 µm, 7.8 x 300 mm
mobile phase:	0.2 M potassium phosphate, 0.45 M KCl, pH 7.0
gradient:	isocratic
flow rate:	1.0 mL/min
run time:	30 min
temp:	ambient
LC:	HP-1090
detection	214, 280 nm
injection:	15 µg

Cation Exchange HPLC

column:	Tosohaas TSK SP-5PW, 10 µm, 7.5 x 75mm

mobile phase:	A: 10 mM sodium phosphate, 10% (v/v) AcCN, pH 7.0
	B: A + 1 M ammonium chloride
gradient:	10 - 40% B (60'), 40-60% B (5'), 60-10%B (1'), 71-86% B (15')
flow rate:	0.5 mL/min
run time:	86 min
temp:	35°C
LC:	HP-1090
detection	214 nm
injection:	15 µg

C. ELISA. This assay with a range of 0.39 - 6.25 ng/mL was carried out by Immunoassay Services (Test Procedure Code SNGF:1 of Genentech, Inc.). Each rhNGF sample was diluted in assay diluent to two target concentrations of 5 and 2.5 ng/mL, and each dilution was submitted in micronic tubes in triplicate. The protein concentration in mg/mL was normalized to a -70°C internal reference standard which was submitted for the same assay.

D. Radioreceptor Assay (RRA). This assay measures the ability of unlabeled rhNGF to compete with 125I-rhNGF for receptor binding on PC-12 cells. This assay was carried out by Bioassay Service (Genentech, Inc. Test Procedure SNGF:6) and has a range of 3-80 ng/mL. Each rhNGF sample was diluted in assay diluent to two target concentrations of 25 and 12.5ng/mL, and each dilution was submitted in micronic tubes in duplicate. The protein concentration in mg/mL was normalized to a -70°C internal reference standard which was submitted for the same assay.

E. PC-12 Cell Survival Bioassay. This assay determines the ability of rhNGF to bind to its receptors and generate intracellular signals that result in the survival of PC-12 cells under serum-free culture conditions. This assay was carried out by Bioassay Service (Test Procedure SNGF:7) and has a range of 0.24-30 ng/mL. The active protein concentration in mg/mL was normalized to a -70°C internal reference standard which was submitted for the same assay.

F. Visual Inspection. Visual inspection was performed on all formulations in vials at the time of sampling. Samples were observed for solution clarity, color, opalescence and particulate formation.

G. pH Determination. pH of all formulations was determined at each timepoint using a radiometer (model PHM82, Radiometer America Inc.) and a micro-electrode (model M1-410, Microelectrodes, Inc.). Standard solutions of pH 4.01 and pH 7.00 were used for the standardization and calibration of the radiometer prior to pH measurement.

H. Preservative Effectiveness Test. The lead rhNGF multi-dose liquid formulations which were stable at 5°C for 6 months were sent to Northview Lab for bacterial challenge testing based on USP and EP standard criteria.

I. Circular Dichroism (CD) Analysis. An AVIV® spectropolarimeter Model 60 DS equipped with water bath and data processor was used to measure circular dichroism. Measurements were made at 20°C. Quartz cuvettes of 1.0 cm cell path length was used for measuring near-UV CD. The CD spectra was taken at 0.2 nm intervals, with a 0.5 nm bandwidth, and 3.0 second averaging time. Each sample for CD measurement was taken continuously for 24 hours. The CD data were expressed as the mean residue ellipticity [q], degree.cm2/decimole, using the mean residue weight of 120 for rhNGF.

Results

A preservative screening study was first performed to examine the physical compatibility of several commonly used preservatives with rhNGF at 0.1 mg/mL in the 20 mM sodium acetate formulation at pH 5.5. These preservatives include benzyl alcohol, phenol, m-cresol, methylparaben and propylparaben. In addition, the physical compatibility of these preservatives with rhNGF in the acetate formulation with the presence of surfactants such as polysorbate 20 and pluronic acid (F68) was also studied. The physical compatibility results are shown in Table 4.

Table 4: List of rhNGF Liquid Formulations Selected for Long Term Stability Testing

I. Current liquid formulation
   1. 2 mg/mL rhNGF in 10 mM acetate, 142 mM sodium chloride, pH 5.5

II. Control liquid formulations (no preservative)
   1. 0.1 mg/mL rhNGF in 20 mM acetate, 136 mM sodium chloride, pH 5.5
   2. 0.1 mg/mL rhNGF in 20 mM acetate, 136 mM sodium chloride, 0.01% F68, pH 5.5

III. Multi-dose liquid formulations
   1. 0.1 mg/mL rhNGF in 20 mM acetate, 136 mM sodium chloride, 0.9% benzyl alcohol, pH 5.5
   2. 0.1 mg/mL rhNGF in 20 mM acetate, 136 mM sodium chloride, 0.25% phenol, pH 5.5
   3. 0.1 mg/mL rhNGF in 20 mM acetate, 136 mM sodium chloride, 0.01% F68, 0.9% benzyl alcohol, pH 5.5
   4. 0.1 mg/mL rhNGF in 20 mM acetate, 136 mM sodium chloride, 0.01% F68, 0.25% phenol, pH 5.5

Among the preservatives used for screening, they are all physically compatible with rhNGF at 0.1 mg/mL in the acetate formulation at pH 5.5. In the presence of polysorbate 20 at 0.01% in the same formulation, only benzyl alcohol and phenol at final concentrations of 0.9% and 0.25% respectively were physically compatible with rhNGF. Phenol at 0.45% and m-cresol at 0.25% each formed a cloudy solution with rhNGF in the acetate formulation in the presence of polysorbate 20. The rhNGF solution also became slightly opalescent upon the addition of methylparaben at 0.18% or propylparaben at 0.02% to the polysorbate 20 containing acetate formulation. On the other hand, pluronic acid at 0.01% in the same formulation did not cause any physical incompatibility between rhNGF and all the preservatives tested.

Based on the preservative screening study results, several rhNGF multi-dose liquid formulations containing either 0.9% benzyl alcohol or 0.25% phenol in 20 mM acetate at pH 5.5 with

and without 0.01% F68 were set up for long term stability study. A list of these formulations were given in Table 5.

Table 5: Physical Compatibility of Preservatives with 0.1 mg/mL rhNGF Liquid Formulations

Formulation buffer	Surfactant	Preservative	Results
20 mM acetate, pH 5.5	none	0.9% benzyl alcohol	co/cl
136 mM NaCl		0.25% phenol	co/cl
		0.45% phenol	co/cl
		0.25% m-cresol	co/cl
		0.18% methylparaben	co/cl
		0.02% propylparaben	co/cl
20 mM acetate, pH 5.5	0.01% Tween 20	0.9% benzyl alcohol	co/cl
136 mM NaCl		0.25% phenol	co/cl
		0.45% phenol	cloudy
		0.25% m-cresol	cloudy
		0.18% methylparaben	sl. opal
		0.02% propylparaben	sl. opal
20 mM acetate, pH 5.5	0.01% F68	0.9% benzyl alcohol	co/cl
136 mM NaCl		0.25% phenol	co/cl
		0.45% phenol	co/cl
136 mM NaCl		0.25% m-cresol	co/cl
		0.18% methylparaben	co/cl
		0.02% propylparaben	co/cl

Stability of rhNGF in these formulations was assayed by the following techniques: SE-HPLC, RP-HPLC, IE-HPLC, ELISA, radioreceptor assay (RRA), PC-12 cell survival bioassay, pH, and visual inspection. The acceptability of a multi-dose liquid formulation for rhNGF will be based on comparison to the current liquid formulation which consists of 2 mg/mL rhNGF in 10 mM sodium acetate at pH 5.5, and 142 mM sodium chloride. In the other word, the preserved formulation should be as stable as the current liquid formulation. Results obtained to date represent 12 months at -70 and 5°C, 9 months at 25°C, and 3 months at 40°C stability monitoring data.

Size-Exclusion Chromatography. Size-exclusion HPLC was employed to detect and quantitate aggregate formation in the rhNGF multi-dose liquid formulations as well as their control formulations which contain no preservative. Using this technique, rhNGF elutes as dimer (main peak) at a retention time of 8.6 minutes. Benzyl alcohol and phenol elute at 16 and 19 minutes respectively. The appearance of leading shoulder on the dimer main peak indicates the presence of aggregate of higher molecular weight. The data in Table 6 shows that rhNGF is stable to aggregate formation in all formulations containing 0.9% benzyl alcohol as preservative.

Table 6: Effect of preservative on aggregation of 0.1 mg/mL rhNGF in liquid formulations was determined by SEC-HPLC. Samples were stored at 5°C for 12 months, 25°C for 9 months and 40°C for 3 months.

Formulation buffer	Surfactant	Preservative	% Aggregate		
			5°C	25°C	40°C
10 mM acetate, pH 5.5 145 mM NaCl, 2 mg/mL	none	none	0	0.2	0.4
20 mM acetate, pH 5.5 136 mM NaCl, 0.1mg/mL	none	none	0	0	0
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	0.9% benzyl alc.	0	0	0
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	0.25% phenol	0	0.4	0.5
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	none	0	0	0
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	0.9% benzyl alc.	0	0	0
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	0.25% phenol	0	0.5	0.5

A small amount of aggregate (less than 1%) was detected in the phenol containing formulations (with and without 0.01% F68 as surfactant) after 3 months at 40°C and 9 months at 5°C. Total protein recovery of these samples, compared to their -70°C controls, was given in Table 7.

Table 7: Quantitation of total rhNGF by SE-HPLC. Samples were stored at 5°C for 12 months, 25°C for 9 months and 40°C for 3 months.

Formulation buffer	Surfactant	Preservative	% Recovery		
			5°C	25°C	40°C
10 mM acetate, pH 5.5 145 mM NaCl, 2 mg/mL	none	none	102	102	102
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	none	101	101	101
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	0.9% benzyl alc.	102	99	101
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	0.25% phenol	99	97	98
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	none	101	101	98
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	0.9% benzyl alc.	101	99	99

20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	0.25% phenol	100	97	97

Current formulation, controls and benzyl alcohol containing formulations had 99% or greater protein recovery after 9 months at 25°C, while phenol containing formulations had 97% for the same storage time and temperature. These results indicate that rhNGF is more compatible and stable with benzyl alcohol than phenol in all formulations studied.

Reversed-Phase HPLC. The rhNGF used in this study consists of mainly 118/118 homodimer and a small amount of 120/120 homodimer. Under the conditions of reversed-phase chromatography, the two rhNGF dimeric forms are dissociated and their monomers are separated. RP-HPLC separates the rhNGF monomers based on the hydrophobicity of each species. The 118 monomer which is more hydrophobic than the 120 monomer elutes at a retention time of 23 minutes. The 120 monomer elutes as a small peak in front of the 118 monomer peak. Comparison of RP-HPLC chromatograms of rhNGF in the benzyl alcohol preserved formulation containing no surfactant at 5, 25, and 40 °C are shown in Figure 8. The degradation of rhNGF stored at elevated temperatures was mainly due to the formation of iso-aspartate, loss in 118 and 120 monomer peak areas, clip formation and increase in misfolded rhNGF as determined by RP-HPLC. The mono- and di- oxidized rhNGF peaks and the deamidated rhNGF peak remain unchanged. In this study, rhNGF is defined as the sum of the 118 and 120 monomer peak areas by RP-HPLC, and the results are reported as percent rhNGF remaining as compared to the -70°C controls.

Decrease in percent protein remaining due to the loss of 118 and 120 monomer peak areas assayed by RP-HPLC is the major degradation for rhNGF in liquid formulation. At 5°C, the stability of rhNGF in multi-dose formulations as determined by RP-HPLC are essentially equivalent to the non-preserved control formulations as well as the current formulation (more than 95% rhNGF remaining after 12 months) except for the phenol preserved formulation containing 0.01% F68 (Figure 9). This formulation had slightly less percent rhNGF remaining (93%) after 12 months at 5°C. At 25°C, rhNGF is obviously less stable in the presence of 0.25% phenol than 0.9% benzyl alcohol as preservative in the 20 mM acetate formulation at pH 5.5 (Figure 10). The combination of phenol and F68 in the acetate formulation caused more degradation of the protein than the presence of phenol alone.

Iso-aspartate formation of rhNGF in liquid form is time and temperature dependent. The rate of iso-aspartate formation increases with increase in time and temperature. At 5°C, all formulations show a similar rate of iso-aspartate formation (Figure 11). There was about 1.5% iso-aspartate formed in all rhNGF multi-dose formulations and their non-preserved control formulations after 12 months at 5°C. However, the rate of iso-aspartate formation is slightly higher in the rhNGF formulations preserved with 0.9% benzyl alcohol than the control formulations and phenol preserved formulations stored at 25°C (Figure 12). Since iso-aspartate formation of rhNGF does not affect the bioactivity of the protein, the effect of preservative on iso-aspartate formation of rhNGF is not a major concern.

Cation Exchange Chromatography. IE-HPLC chromatograms for rhNGF in the current formulation at 3 months at 5, 25, and 40°C are shown in Figure 13. There are three major peaks observed. The predominant peak is the 118/118 dimer (peak b) which elutes at about 48 minutes. The peak c behind the main peak is from

a serine to glycine substitution at position 1 in one of the two dimer chain. The peak a in front of the main peak is believed to be the oxidized 118/118 and oxidized N-terminally clipped rhNGF. At elevated temperatures (25 and 40°C), degradation of rhNGF as determined by IE-HPLC is characterized by the decrease in peak areas of the 118/118 main peak and the serine to glycine substituted 118/118 dimer and the increase in peak a area. In this study, rhNGF is defined as the sum of the 118/118 dimer (peak b) and one chain serine to glycine dimer (peak c) peak areas by IE-HPLC, and the results are reported as percent rhNGF remaining as compared to the -70°C controls.

Figures 14 and 15 show the percent rhNGF remaining in all rhNGF formulations by IE-HPLC after 12 months at 5°C and 9 months at 25°C, respectively. At 5°C, the peak area of peaks b and c for all rhNGF formulations remained unchange after 12 months. At 25°C, all rhNGF formulations show a similar rate of degradation, and there was no significant difference in stability between the multi-dose formulations and the control formulations as assessed by IE-HPLC.

ELISA. The data in Table 8 show the percent rhNGF remaining at 5, 25 and 40°C after 12, 9 and 3 months of storage, respectively.

Table 8: Stability of current and selected multi-dose liquid formulations for rhNGF determined by ELISA after 12 months at 5°C, 9 months at 25°C, and 3 months at 40°C.

Formulation buffer	Surfactant	Preservative	% rhNGF [a] Remaining		
			5°C	25°C	40°C
10 mM acetate, pH 5.5 145 mM NaCl, 2 mg/mL	none	none	101.2	89.1	102.2
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	none	97.8	102.0	94.4
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	0.9% benzyl alc.	103.1	92.9	97.1
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	0.25% phenol	111.3	88.5	91.6
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	none	98.5	102.7	92.7
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	0.9% benzyl alc.	101.9	92.6	87.7
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	0.25% phenol	103.4	92.5	82.6

a Calculated as a percentage of assay response for -70°C control sample at the same storage period.

Results were normalized to the -70°C controls stored at the same temperature for the same period of time. There were no significant difference between the benzyl alcohol and phenol preserved formulation either in the presence or absence of 0.01% F68 as surfactant for all temperatures and time points studied.

Radioreceptor Binding Activity (RRA). The RRA results are presented in Table 9 and are normalized to the -70°C controls.

Table 9: Stability of current and selected multi-dose liquid formulations for rhNGF determined by RRA after 12 months at 5°C, 9 months at 25°C, and 3 months at 40°C.

Formulation buffer	Surfactant	Preservative	% rhNGF [a]Remaining		
			5°C	25°C	40°C
10 mM acetate, pH 5.5 145 mM NaCl, 2 mg/mL	none	none	111.3	121.5	74.9
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	none	100.6	106.5	82.1
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	0.9% benzyl alc.	94.2	91.3	81.6
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	0.25% phenol	82.0	72.5	68.8
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	none	92.9	79.2	80.8
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	0.9% benzyl alc.	92.0	80.7	83.2
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	0.25% phenol	98.0	83.7	73.7

a Calculated as a percentage of assay response for -70°C control sample at the same storage period.

In the absence of 0.01% F68 in the acetate formulation at pH 5.5, the phenol preserved formulation had less percent protein remaining than both the benzyl alcohol preserved formulation and the control formulation for all temperatures studied. In the presence of 0.01% F68 in the acetate formulation at pH 5.5, rhNGF in the preserved (benzyl alcohol or phenol) and the control formulation had lost about 20% of its bioactivity at 25 and 40°C after 9 and 3 months, respectively. These results suggest that phenol and F68 can affect the ability of rhNGF to bind to the NGF receptor on PC-12 cells. Therefore, benzyl alcohol at 0.9% is a better choice of preservative for rhNGF in the acetate formulation containing no surfactant for multi-use purpose.

PC-12 Cell Survival Bioassay. In contrast to the RRA results, the PC-12 cell survival bioassay data in Table 10 show that there was no significant difference in potency of rhNGF in all formulations stored at 5°C for 12 months and 25°C for 9 months.

Table 10: Stability of current and selected multi-dose liquid formulations for rhNGF determined by bioassay after 12 months at 5°C and 9 months at 25°C.

Formulation buffer	Surfactant	Preservative	% rhNGF [a] Remaining	
			5°C	25°C
10 mM acetate, pH 5.5 145 mM NaCl, 2 mg/mL	none	none	101.7	96.1
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	none	84.3	113.7
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	0.9% benzyl alc	102.2	97.3
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	none	0.25% phenol	95.3	102.1
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	none	101.3	95.9
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	0.9% benzyl alc.	96.6	94.2
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	0.01% F68	0.25% phenol	97.8	96.4

a Calculated as a percentage of assay response for -70°C control sample at the same storage period.

The protein was found to be fully active in all formulations as determined by this bioassay. Therefore, the radioreceptor binding assay is a more stability indicating assay than the cell survival bioassay in determining the bioactivity of rhNGF.

Solutions of all rhNGF formulations were clear and colorless to the naked eyes (Table 11). Particulates were not observed in any of the formulations at all temperatures and timepoints.

Table 11: pH and visual clarity of rhNGF formulations after 12 months at 5°C and 9 months at 25°C.

Formulation buffer	pH 5°C	Visual Clarity 5°C	pH 25°C	Visual Clarity 25°C
10 mM acetate, pH 5.5 145 mM NaCl, 2 mg/mL	5.50	co/cl	5.40	co/cl
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL	5.54	co/cl	5.41	co/cl
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL 0.9% benzyl alc.	5.52	co/cl	5.58	co/cl
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL 0.25% phenol	5.49	co/cl	5.60	co/cl

20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL 0.01% F68	5.47	co/cl	5.53	co/cl
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL 0.01% F68, 0.9% benzyl alc.	5.42	co/cl	5.42	co/cl
20 mM acetate, pH 5.5 136 mM NaCl, 0.1 mg/mL 0.01% F68, 0.25% phenol	5.48	co/cl	5.41	co/cl

co/cl = colorless and clear

pH Results. rhNGF formulated in 10 mM acetate, 142 mM sodium chloride at either pH 5.0 or pH 5.8 had an increase in pH by 0.2 units during the stability study. The multi-dose formulations and their control formulations used in this study were formulated in 20 mM acetate at pH 5.5 which should provide a higher buffer capacity to prevent pH change. Table 11 shows that pH remained unchange for all formulations studied.

Preservative Effectiveness Test. After 6 months of stability study, the most stable multi-dose formulation for rhNGF which consists of 0.1 mg/mL rhNGF in 20 mM acetate at pH 5.5, 136 mM sodium chloride, and 0.9% benzyl alcohol was submitted for preservative efficacy testing. This lead formulation passed both the USP and EP (criteria A and B) after 6 months storage at 5°C.

Circular Dichroism (CD) Analysis. The presence of 0.9% benzyl alcohol in various liquid interferon-gamma (rhIFN-g) formulations induces loss in circular dichroism signals in the near-UV region. The near-UV CD signal of rhIFN-g disappeared within 24 hours, indicating that there was a change in tertiary structure of the protein due to the presence of benzyl alcohol. However, this phenomenon was not observed in the rhNGF formulation preserved with 0.9% benzyl alcohol. After 24 hours of the addition of the preservative, the near-UV CD spectrum remained unchange, suggesting that there is no interaction between rhNGF and benzyl alcohol in the acetate formation at pH 5.5. Figure 16 shows the near-UV CD spectrum of rhNGF, and Figure 17 compares the near-UV CD spectra of rhNGF in the presence and absence of benzyl alcohol after 24 hours at 25°C. Due to the interference of benzyl alcohol at wavelength below 275 nm, CD spectrum of rhNGF was scanned from 325 nm to 275 nm when the sample contained the preservative.

Stresses Testing Stability

1. Agitation Studies. Shaker studies were performed to determine whether it is necessary to add surfactant (F68) in the rhNGF multi-dose formulations at low protein concentration such as 0.1 mg/mL in order to prevent protein aggregation and maintain visual clarity of the solutions during agitation. The Data of Table 12 show that rhNGF at 0.1 mg/mL in the 20 mM acetate formulation at pH 5.5 (with or without preservative) is quite stable to mechanical disruption such as shaking. This suggests that surfactant is not required in formulating rhNGF at 0.1 mg/mL as multi-dose liquid form for stability purpose.

Table: 12: Effect of agitation on stability of rhNGF multi-dose liquid formulations. Samples were shaken at 80 rpm for 6 and 24 hours at room temperature.

Formulation	Hours	% Monomer (SEC)	% Iso-Asp (RP-HPLC)	%NGF (RP-HPLC)	ELISA (mg/mL)	RRA (mg/mL)
1	6	0	0.6	101.6	0.1	0.11
	24	0.4	0.5	101.7	0.09	0.11
2	6	0	0.8	103.6	0.09	0.11
	24	0	0.7	100.8	0.09	0.10
3	6	0	0.6	101.3	0.09	0.10
	24	0	0.6	101.0	0.09	0.10
4	6	0	0.6	100.8	0.09	0.10
	24	0	0.7	101.0	0.09	0.10

Formulations:

1. 2 mg/mL, 10 mM acetate pH 5.5, 145 mM NaCl.
2. 0.1 mg/mL, 20 mM acetate pH 5.5, 136 mM NaCl.
3. 0.1 mg/mL, 20 mM acetate pH 5.5, 136 mM NaCl, 0.9% benzyl alcohol.
4. 0.1 mg/mL, 20 mM acetate pH 5.5, 136 mM NaCl, 0.25% phenol.

2. Freezing-Thawing Studies. Results on the effect of freezing and thawing on stability of 0.1 mg/mL rhNGF multi-dose liquid formulations are presented in Table 13.

Table: 13: Effect of freeze-thaw on stability of rhNGF multi-dose liquid formulations.

Formulation	Freeze -70°C Thaw 5°C	%Aggregate (SEC)	%Iso-Asp (RP-HPLC)	%NGF (RP-HPLC)	ELISA (mg/mL)	RRA (mg/mL)
1	3 cycles	0	0.9	102.1	0.09	0.10
2	3 cycles	0	0.4	102.1	0.08	0.10
3	3 cycles	0	0.8	101.3	0.09	0.11
4	3 cycles	0	0.5	101.8	0.09	0.10

Formulations:

1. 2 mg/mL, 10 mM acetate pH 5.5, 145 mM NaCl.
2. 0.1 mg/mL, 20 mM acetate pH 5.5, 136 mM NaCl.
3. 0.1 mg/mL, 20 mM acetate pH 5.5, 136 mM NaCl, 0.9% benzyl alcohol.
4. 0.1 mg/mL, 20 mM acetate pH 5.5, 136 mM NaCl, 0.25% phenol.

After 3 cycles of freezing and thawing, the 0.1 mg/mL rhNGF in the 20 mM acetate formulation at pH 5.5 as control and the two multi-dose formulations containing either 0.9% benzyl alcohol or 0.25% phenol did not show any loss in stability of the protein. They are as stable as the current 2 mg/mL rhNGF liquid formulation after 3 cycles of freezing and thawing between -70 and 5°C.

3. Light Compatibility Studies. Table 14 summarizes the effect of light on stability of rhNGF in the current 2 mg/mL formulation, the 0.1 mg/mL rhNGF control formulation, and the benzyl alcohol or phenol preserved 0.1 mg/mL rhNGF formulations.

Table 14: Effect of light on stability of rhNGF multi-dose liquid formulations. Samples were illuminated at a light intensity of 20,000 lux at 28°C.

Formulation	Conc. (mg/mL)	Storage Condition	Weeks	%Aggregate (SEC)	ELISA (mg/mL)	RRA (mg/mL)
10 mM acetate pH5.5	2	Dark	2	0	2.20	2.00
145 mM NaCl			5	0.3	2.20	2.00
20 mM acetate pH5.5	0.1	Dark	2	0	0.10	0.11
136 mM NaCl			5	0	0.09	0.10
20 mM acetate pH5.5	0.1	Dark	2	0	0.10	0.11
136 mM NaCl,			5	0	0.10	0.10
0.9% benzyl alcohol						
20 mM acetate pH5.5	0.1	Dark	2	0	0.10	0.10
136 mM NaCl,			5	0.2	0.10	0.10
0.25% phenol						
10 mM acetate pH5.5	2	Light	2	0.4	2.20	2.40
145 mM NaCl			5	1.6	2.00	1.80
20 mM acetate pH5.5	0.1	Light	2	0	0.10	0.10
136 mM NaCl			5	0.3	0.09	0.09
20 mM acetate pH5.5	0.1	Light	2	0	0.10	0.10
136 mM NaCl,			5	0.2	0.09	0.09
0.9% benzyl alcohol						
20 mM acetate pH5.5	0.1	Light	2	0.7	0.09	0.10
136 mM NaCl,			5	12.1	0.07	0.04
0.25% phenol						

After storage for 2 weeks in the light box, there was no significant loss in stability of the protein in all formulations studied. However, after 5 weeks of storage in the light box, SE-HPLC indicated an increase in aggregate formation occurred in the current formulation (1.6%). Aggregate formation was even more pronounced in the phenol preserved formulation (12.1%) after 5 weeks exposure to light. There was also a 30% loss in protein concentration and 60% in bioactivity in the light exposed phenol containing formulation as determined by ELISA and RRA, respectively. Both benzyl alcohol preserved formulation and the 0.1 mg/mL rhNGF control formulation were stable after exposure to light for 5 weeks. All control vials wrapped with aluminum foil were stable after 5 weeks of storage in the light box. These results suggest that rhNGF is more sensitive to light at higher protein concentration (2 mg/mL) than at lower protein concentration (0.1 mg/mL) in the acetate formulation at pH 5.5. In the presence of phenol, rhNGF degrades more faster upon light exposure.

All 0.1 mg/mL rhNGF multi-dose liquid formation at pH 5.5 are stable at 5°C for 12 months. At 25°C, the formulations (with or without F68) using 0.25% phenol as preservative were less stable than the formulations using 0.9% benzyl alcohol.

0.1 mg/mL rhNGF Formulations at pH 5.5 containing surfactant (F68) are as stable as the formulations containing no surfactant.

The lead multi-dose formulation for rhNGF is 0.1 mg/mL protein in 20 mM acetate, pH 5.5, 136 mM NaCl and 0.9% benzyl alcohol filled in 3 cc vial with 1.8 mL filled. This formulation passed both the USP and EP preservative efficacy testing after 6 month storage at 5°C.

rhNGF at 0.1 mg/mL formulated in 20 mM acetate, 136 mM NaCl pH 5.5 is as stable as the current 2 mg/mL liquid formulation.

Agitation has no effect on stability of rhNGF, with regardless to protein concentration or excipient in the formulation.

rhNGF is more stable in the dark than in the light especially if the formulation contains phenol as preservative.

rhNGF at 2 mg/mL in the current formulation and at 0.1 mg/mL in the multi-dose liquid formulations can undergo at least 3 cycles of freezing (-70°C) and thawing (5°C) without any adverse effect on the stability of the protein.

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WHAT IS CLAIMED IS:

1. A pharmaceutical composition, comprising a pharmaceutically effective amount of nerve growth factor and a pharmaceutically acceptable acetate-containing buffer.

2. The composition of claim 1 having a pH from pH 5 to 6.

3. The composition of claim 1, wherein the buffer is sodium acetate.

4. The composition of claim 1 having an acetate concentration of 0.1 to 200 mM.

5. The composition of claim 1, wherein the NGF concentration is 0.07 to 20 mg/ml.

6. The composition of claim 1, further comprising a pharmaceutically acceptable preservative.

7. The composition of claim 7, wherein the preservative is selected from the group consisting of benzyl alcohol,

phenol, m-cresol, methylparaben, and propylparaben.

8. The composition of claim 8, wherein the preservative is benzyl alcohol.

9. The composition of claim 1, wherein the benzyl alcohol concentration is form 0.1 to 2.0%

10. The composition of claim 1, further comprising a pharmaceutically acceptable surfactant.

11. The composition of claim 1, further comprising a physiologically acceptable concentration of sodium chloride.

12. The composition according to claim 1, wherein the nerve growth factor has a concentration of at least about 0.1 mg/ml and said acetate ion has a concentration of 10 mM to 50 mM.

13. The composition according to claim 1, wherein said nerve growth factor has a concentration of 0.1 to about 2.0 mg/ml and said acetate ion has a concentration of 10 mM to 50 mM.

14. The composition of claim 1, wherein the NGF concentration is 0.1 mg/ml, the sodium acetate concentration is 20 mM, the pH is 5.5, the sodium chloride concentration is 136 mM, and benzyl alcohol is 0.9% (v/v).

15. The composition of claim 1, wherein the NGF concentration is 2.0 mg/ml, the sodium acetate concentration is 10 mM, the pH is 5.5, and the sodium chloride concentration is 142 mM.

16. A composition produced by the process comprising formulating nerve growth factor and a pharmaceutically acceptable acetate-containing buffer.

17. The composition of claim 16 wherein the composition is formulated with 0.1 mg/ml, 20 mM sodium acetate, 136 mM sodium chloride, 0.9% (v/v) benzyl alcohol, at pH of 5.5

18. A kit for NGF administration, comprising a vial containing a pharmaceutical composition comprising a pharmaceutically effective amount of nerve growth factor and a pharmaceutically acceptable acetate-containing buffer.

19. The kit of claim 18, wherein the composition volume is from 1.6 to 2.0 ml.

20. The kit of claim 18, wherein the vial reduces light exposure of the composition.

21. The kit of claim 18, wherein the composition is stored from 2 to 8 degrees C.

22. The kit of claim 18, wherein the vial comprises a multi-dose volume of NGF formulation.

23. A method for the preparation of the composition of claim 1 comprising the steps of compounding said NGF and acetate-containing buffer.

24. A method of increasing the stability of NGF in a pharmaceutical composition containing NGF as active principle, comprising incorporating acetate in said composition, wherein said acetate is present in an amount and pH effective to increase the stability of the NGF.

25. The composition of claim 1, wherein the nerve growth factor is 118 amino acid NGF.

26. The composition of claim 25, wherein the nerve growth factor 118/118 rhNGF.

27. The composition of claim 25, wherein the nerve growth factor is secreted from chinese hamster ovary cells.

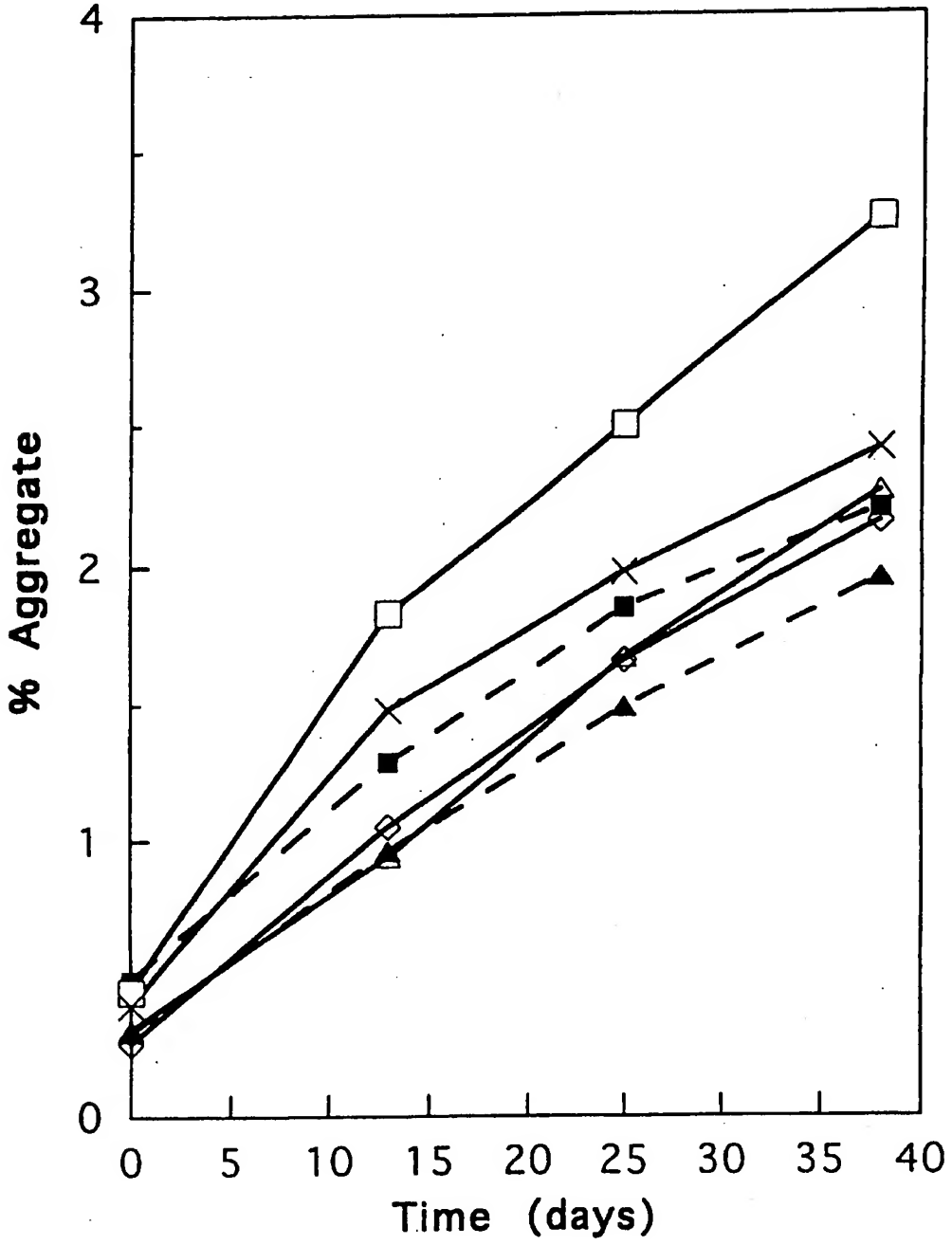


FIG. 1

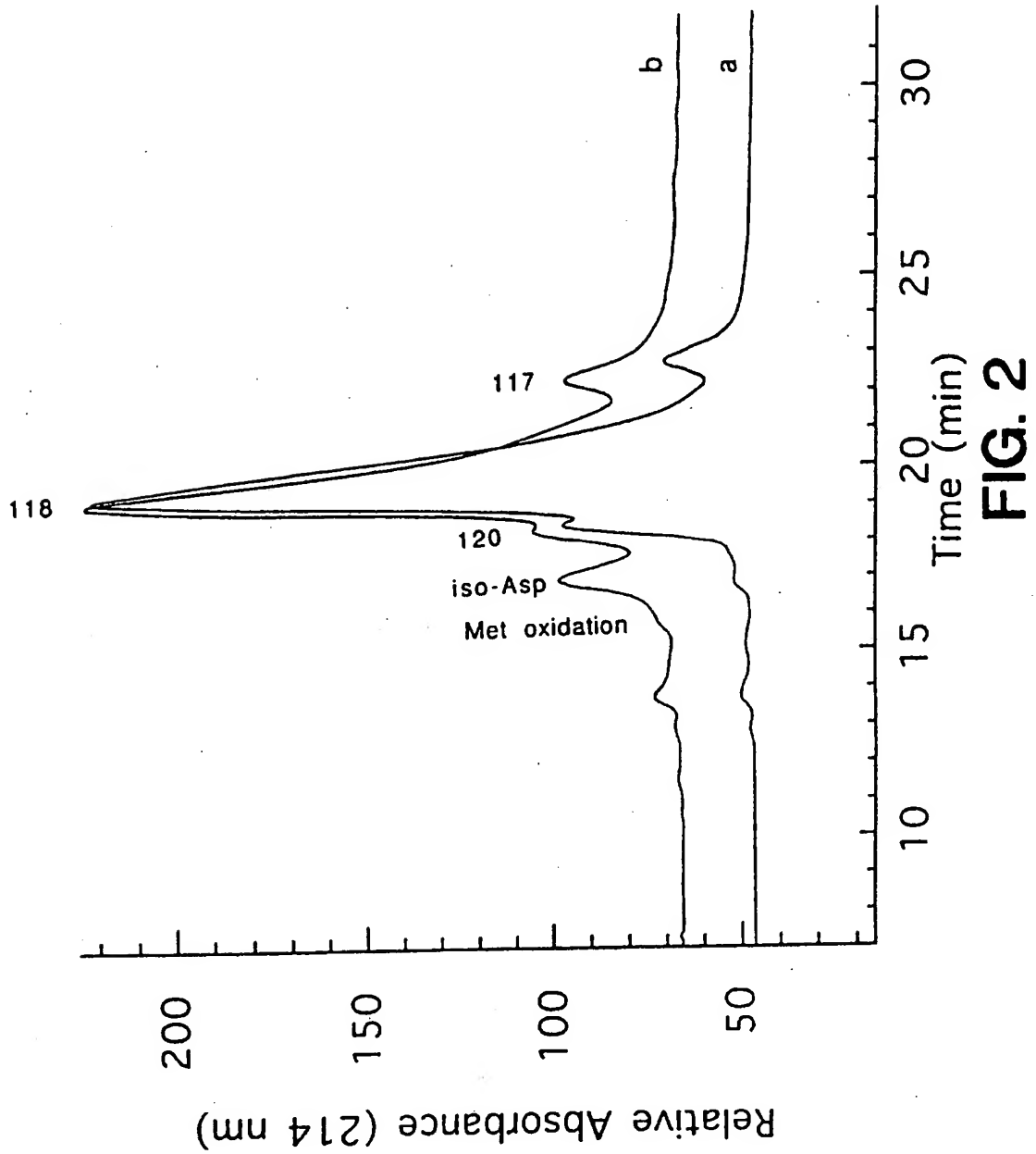

b
a
117
118
120
iso-Asp
Met oxidation
Time (min)
10
15
20
25
30
Relative Absorbance (214 nm)
200
150
100
50

FIG. 2

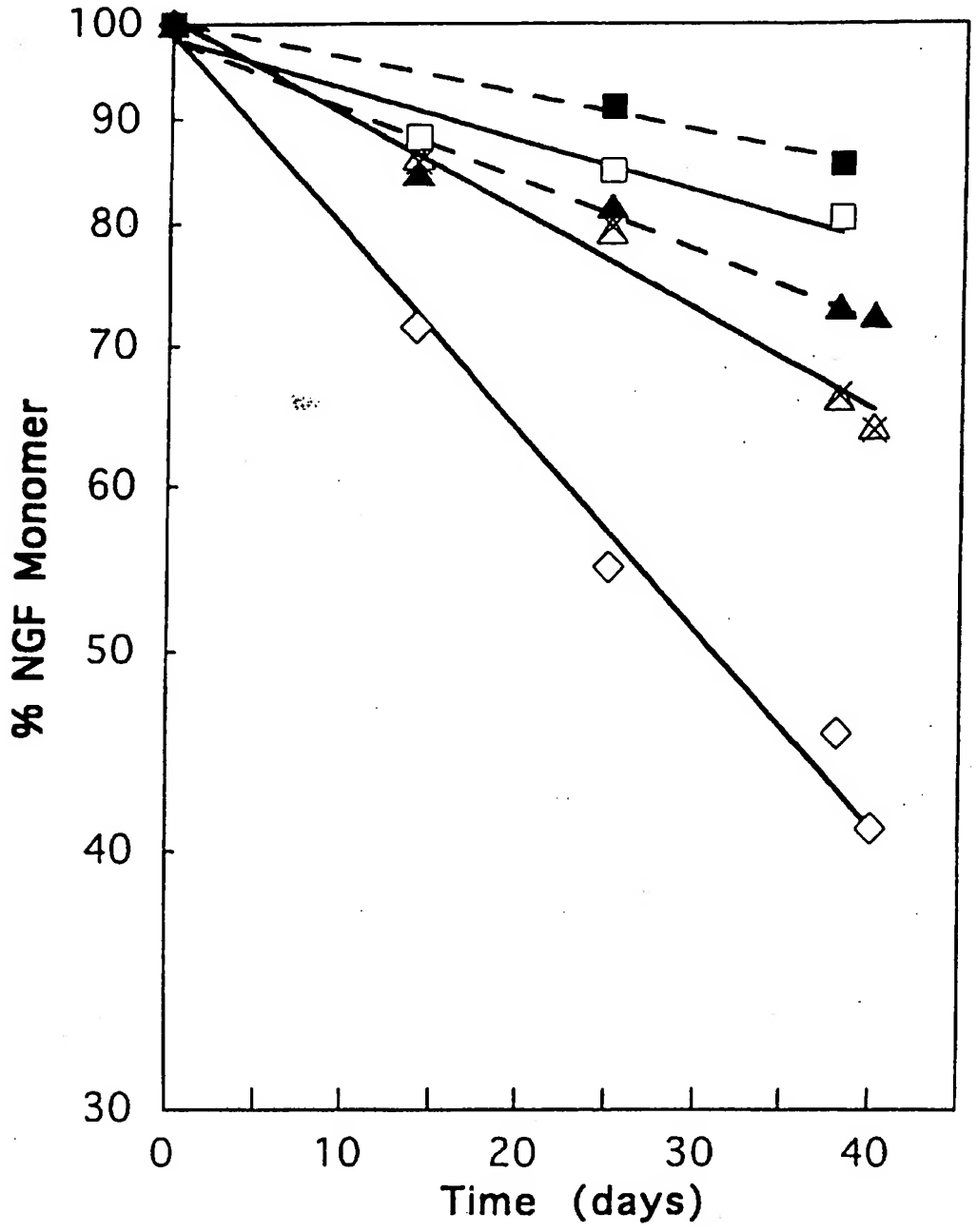


FIG. 3

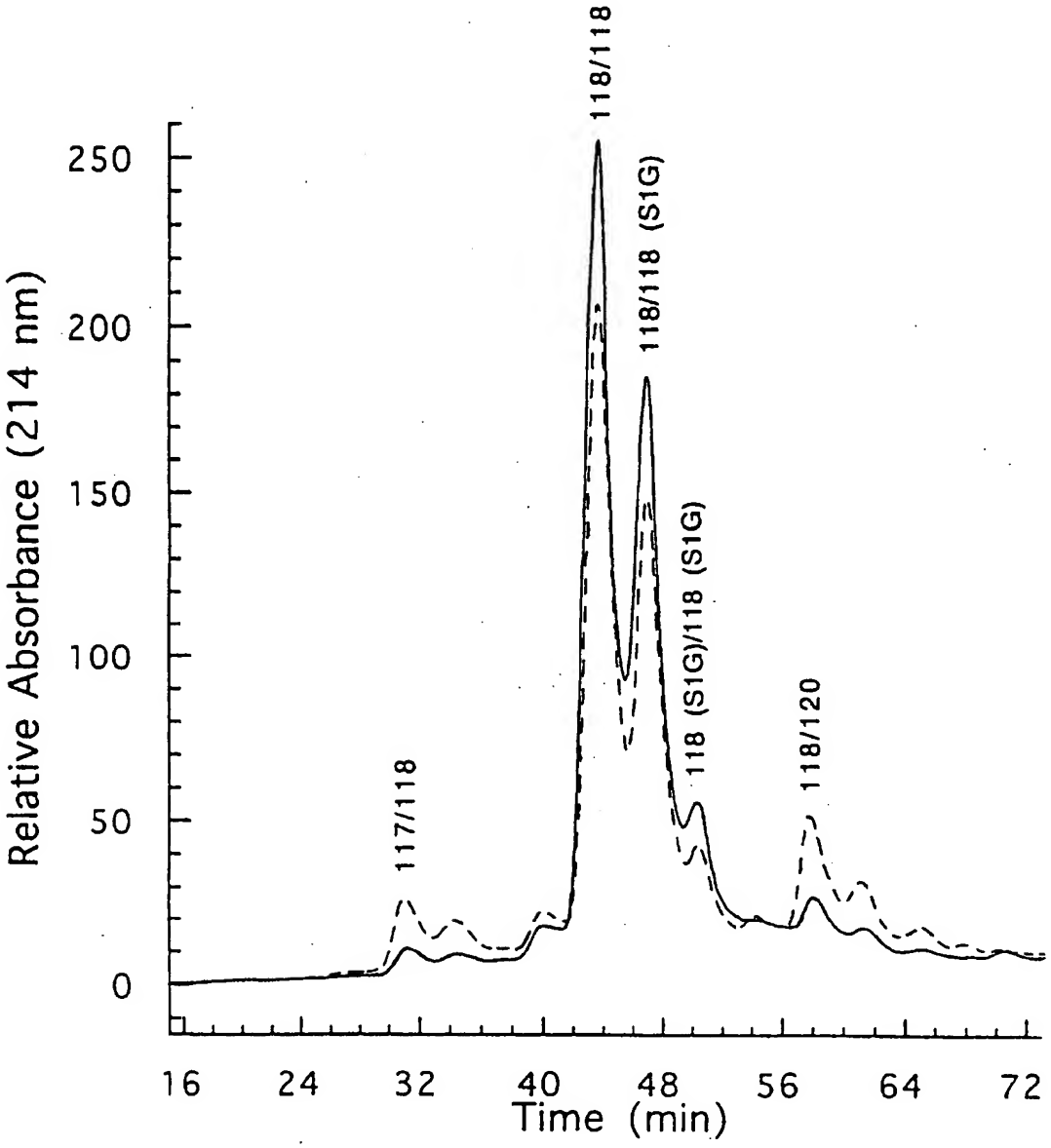

Relative Absorbance (214 nm)
250
200
150
100
50
0
16
24
32
40
48
56
64
72
Time (min)
117/118
118/118
118/118 (S1G)
118 (S1G)/118 (S1G)
118/120

FIG. 4

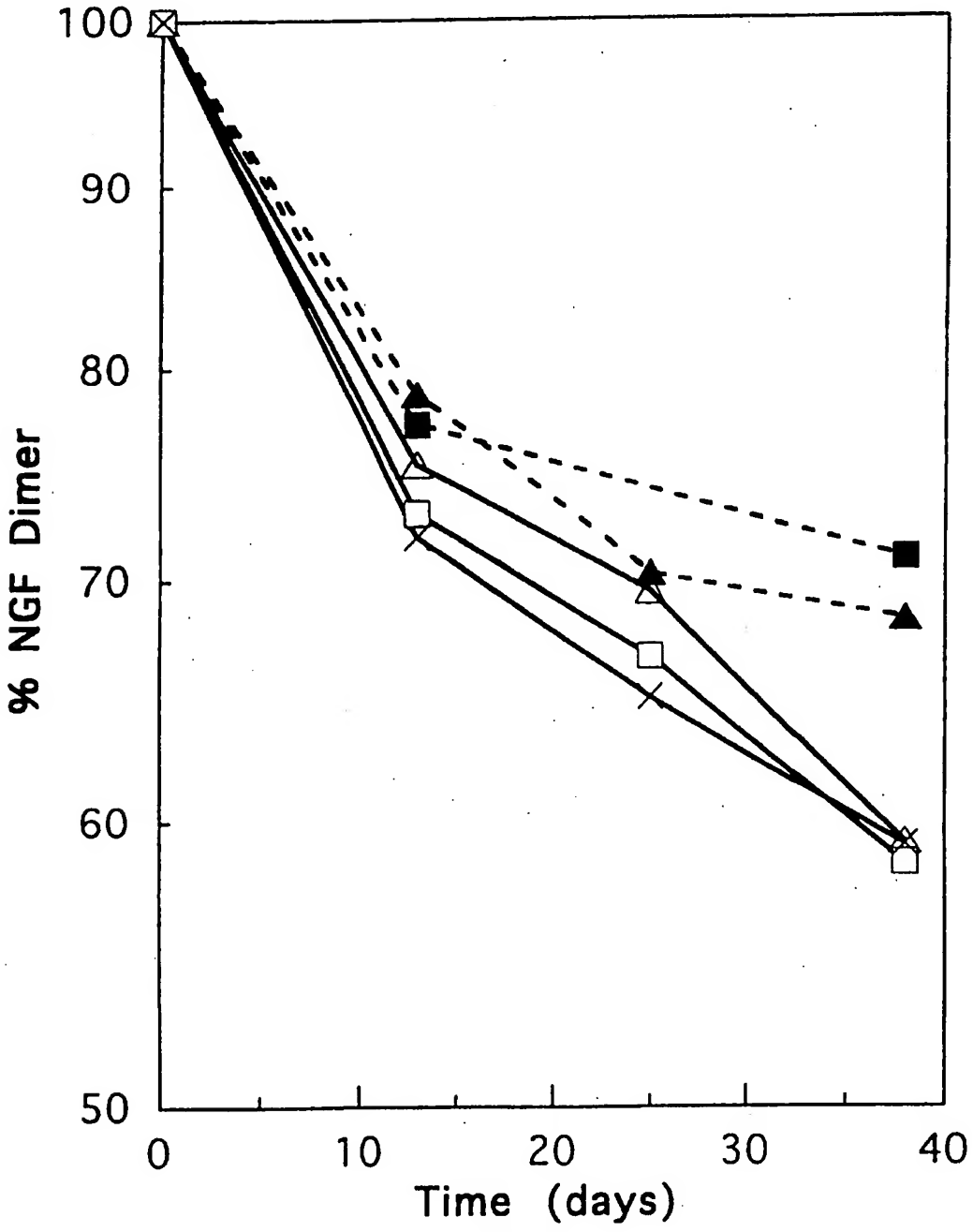


FIG. 5

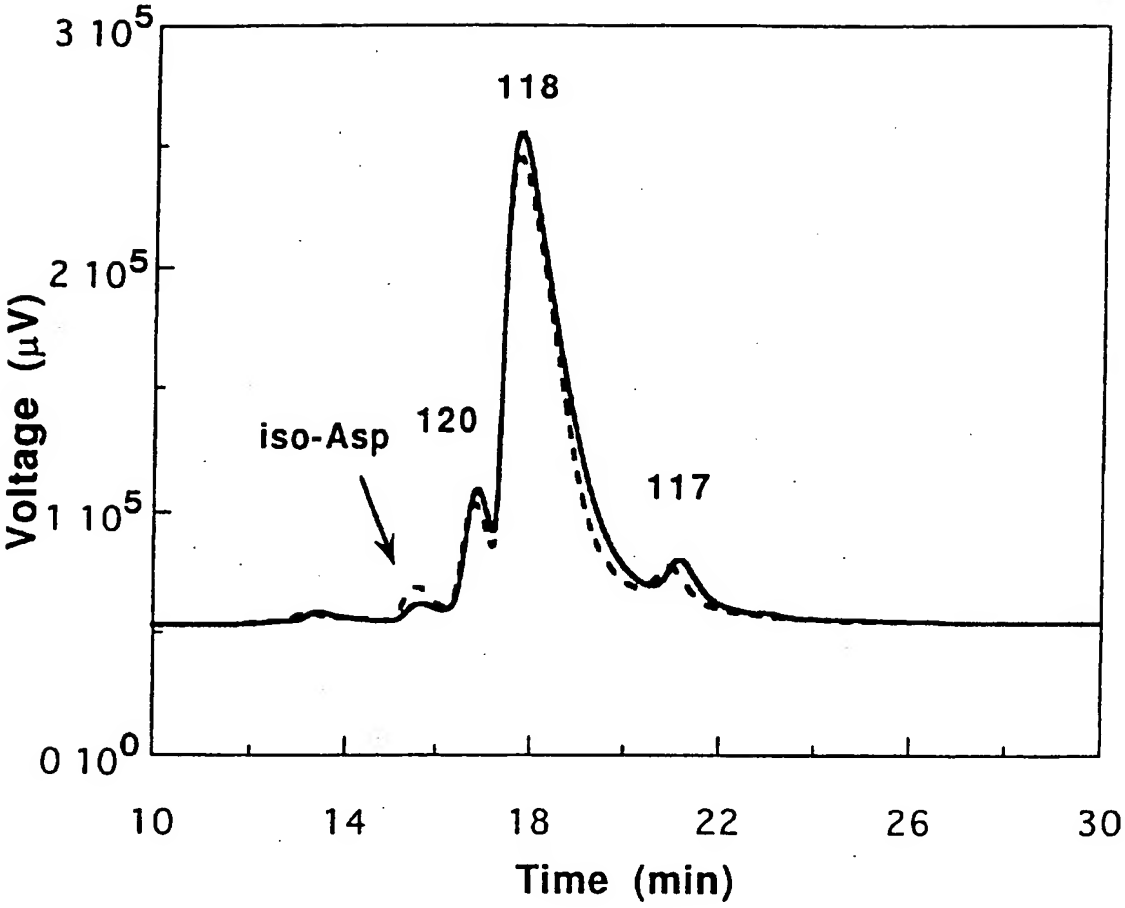

3 10^5
2 10^5
1 10^5
0 10^0
Voltage (μV)
118
120
iso-Asp
117
10
14
18
22
26
30
Time (min)

FIG. 6

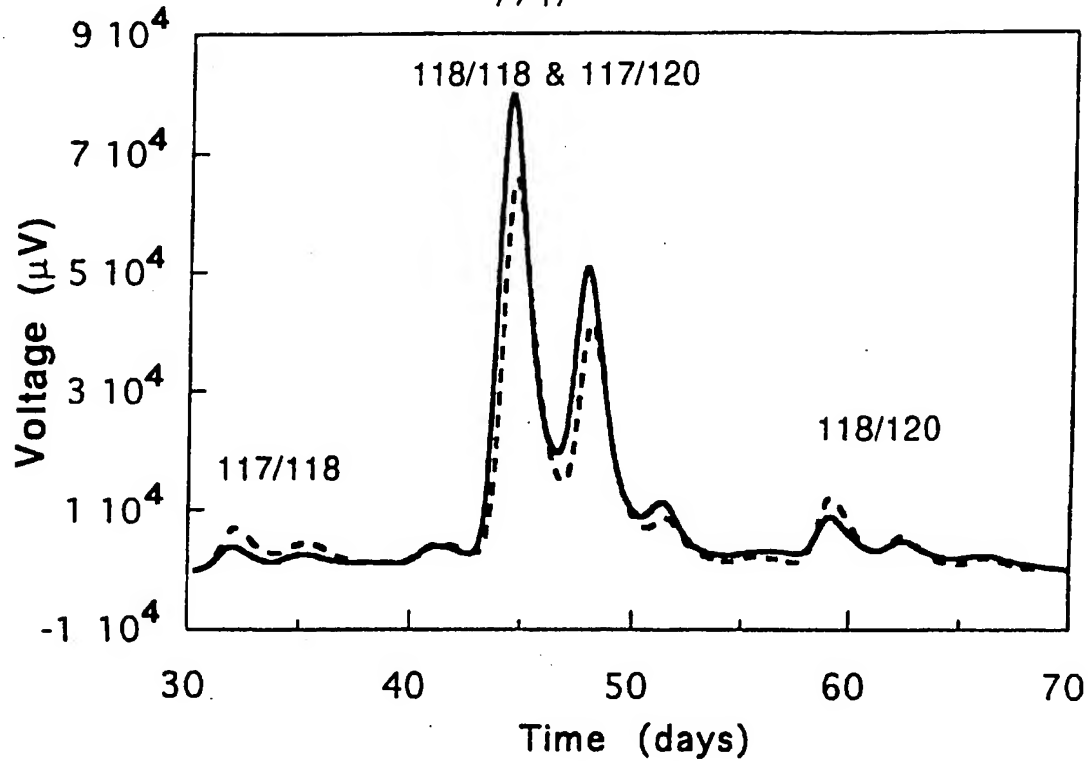

9 10^4
7 10^4
5 10^4
3 10^4
1 10^4
-1 10^4
Voltage (μV)
118/118 & 117/120
117/118
118/120
30
40
50
60
70
Time (days)

FIG. 7A

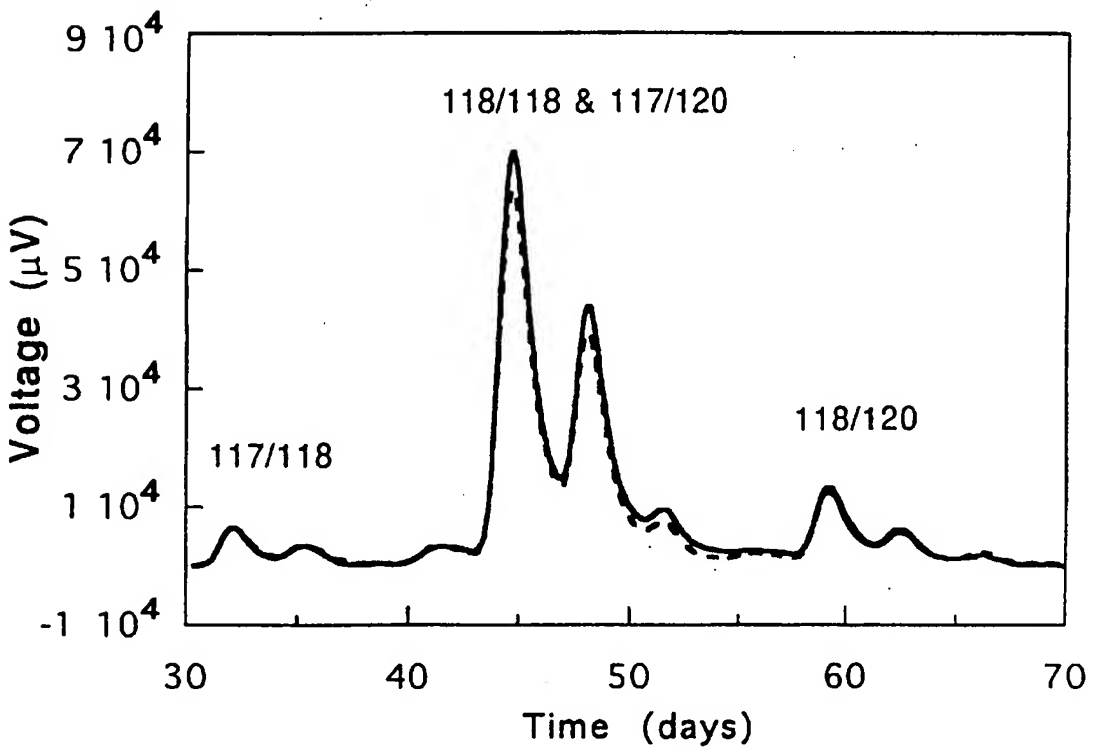
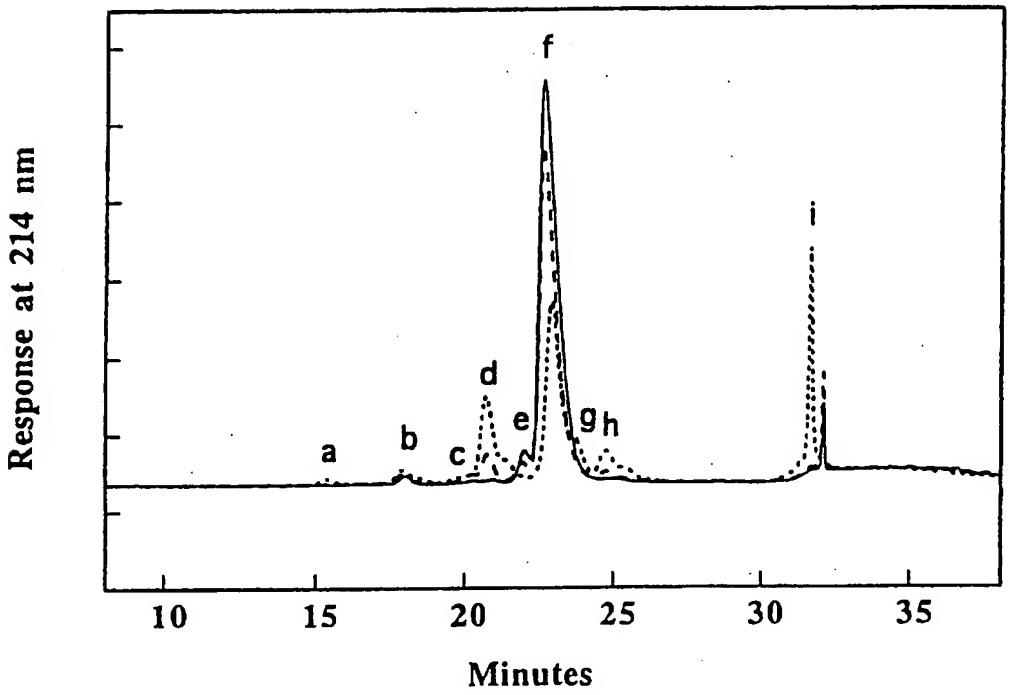

9 10^4
7 10^4
5 10^4
3 10^4
1 10^4
-1 10^4
Voltage (μV)
118/118 & 117/120
117/118
118/120
30
40
50
60
70
Time (days)

FIG. 7B



a = di-oxidized rhNGF
b = deamidated rhNGF
c = mono-oxidized rhNGF
d = Iso-aspartate
e = 120 rh NGF
f = 118 rhNGF
g = N-terminal clipping
h = misfolded rhNGF
i = protein eluted at gradient ramp

FIG. 8

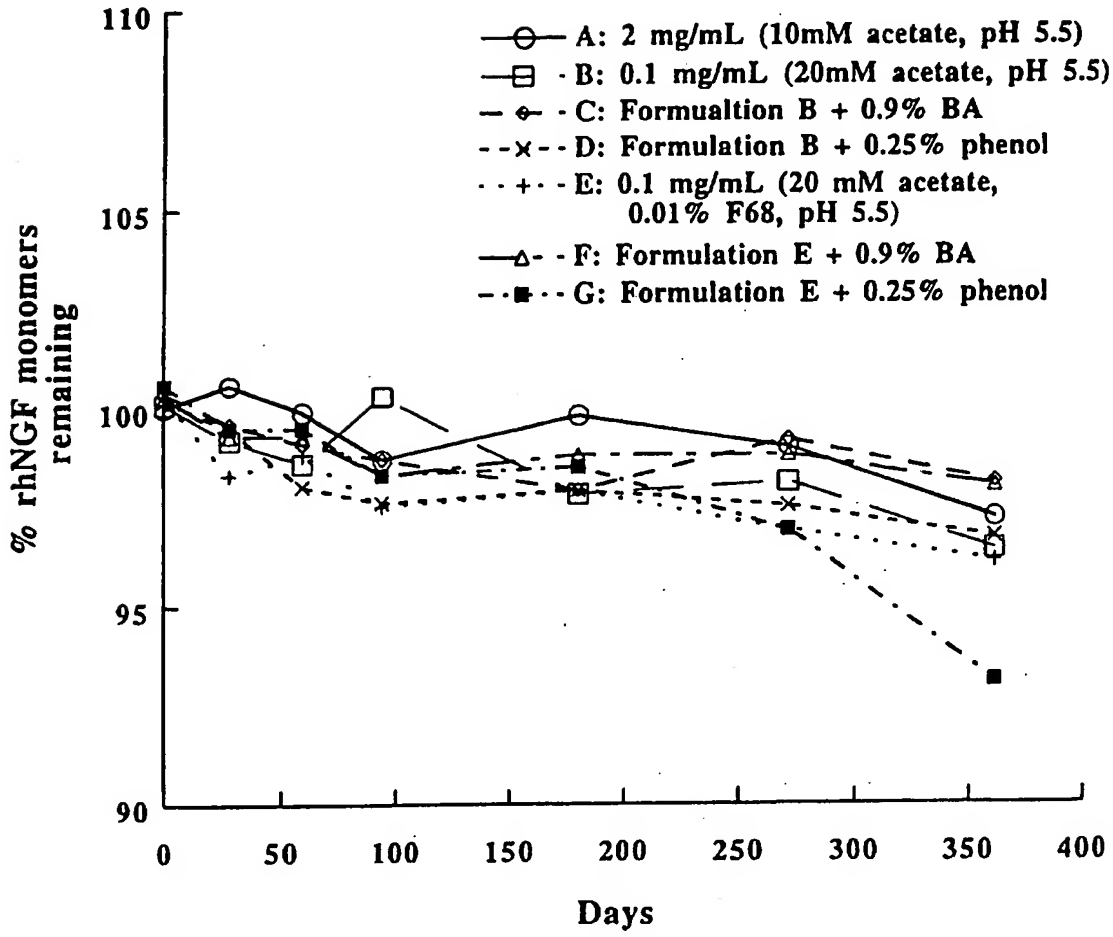

A: 2 mg/mL (10mM acetate, pH 5.5)
B: 0.1 mg/mL (20mM acetate, pH 5.5)
C: Formualtion B + 0.9% BA
D: Formulation B + 0.25% phenol
E: 0.1 mg/mL (20 mM acetate, 0.01% F68, pH 5.5)
F: Formulation E + 0.9% BA
G: Formulation E + 0.25% phenol
% rhNGF monomers remaining
110
105
100
95
90
0
50
100
150
200
250
300
350
400
Days

FIG. 9

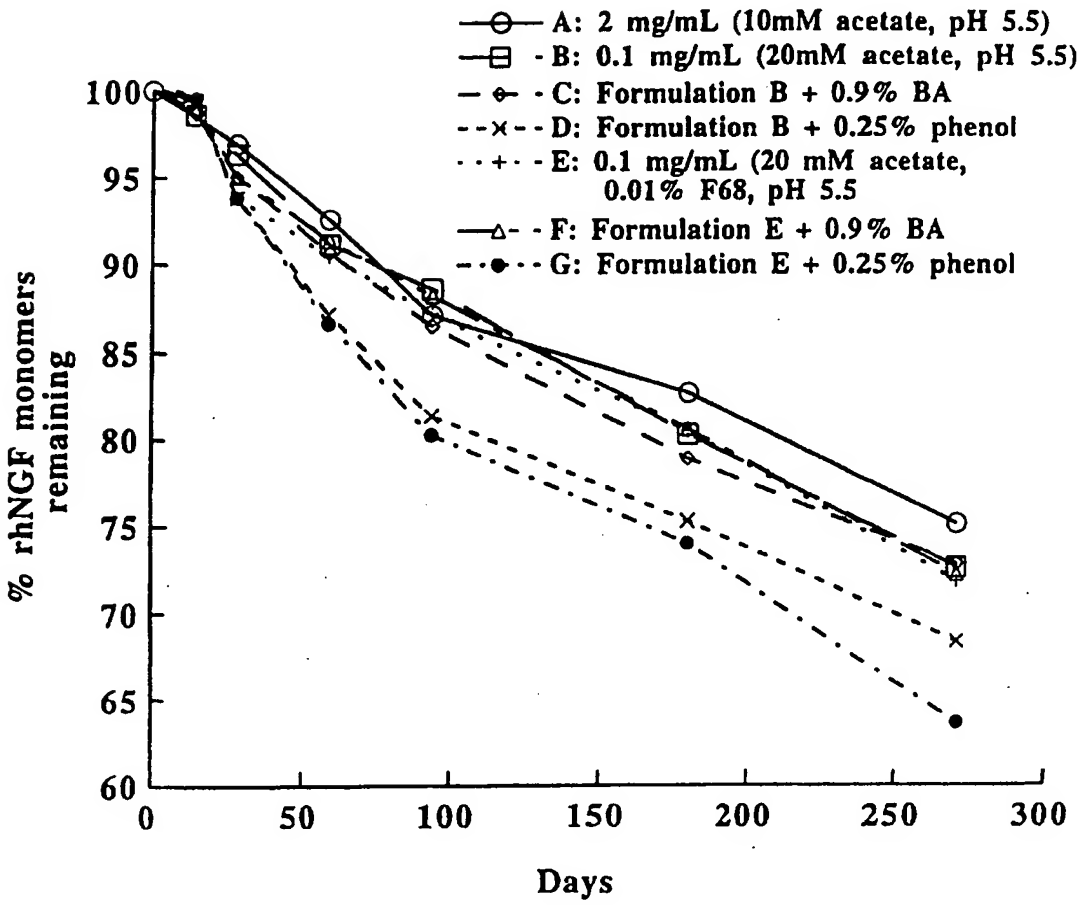

A: 2 mg/mL (10mM acetate, pH 5.5)
B: 0.1 mg/mL (20mM acetate, pH 5.5)
C: Formulation B + 0.9% BA
D: Formulation B + 0.25% phenol
E: 0.1 mg/mL (20 mM acetate, 0.01% F68, pH 5.5
F: Formulation E + 0.9% BA
G: Formulation E + 0.25% phenol
% rhNGF monomers remaining
100
95
90
85
80
75
70
65
60
0
50
100
150
200
250
300
Days

FIG. 10

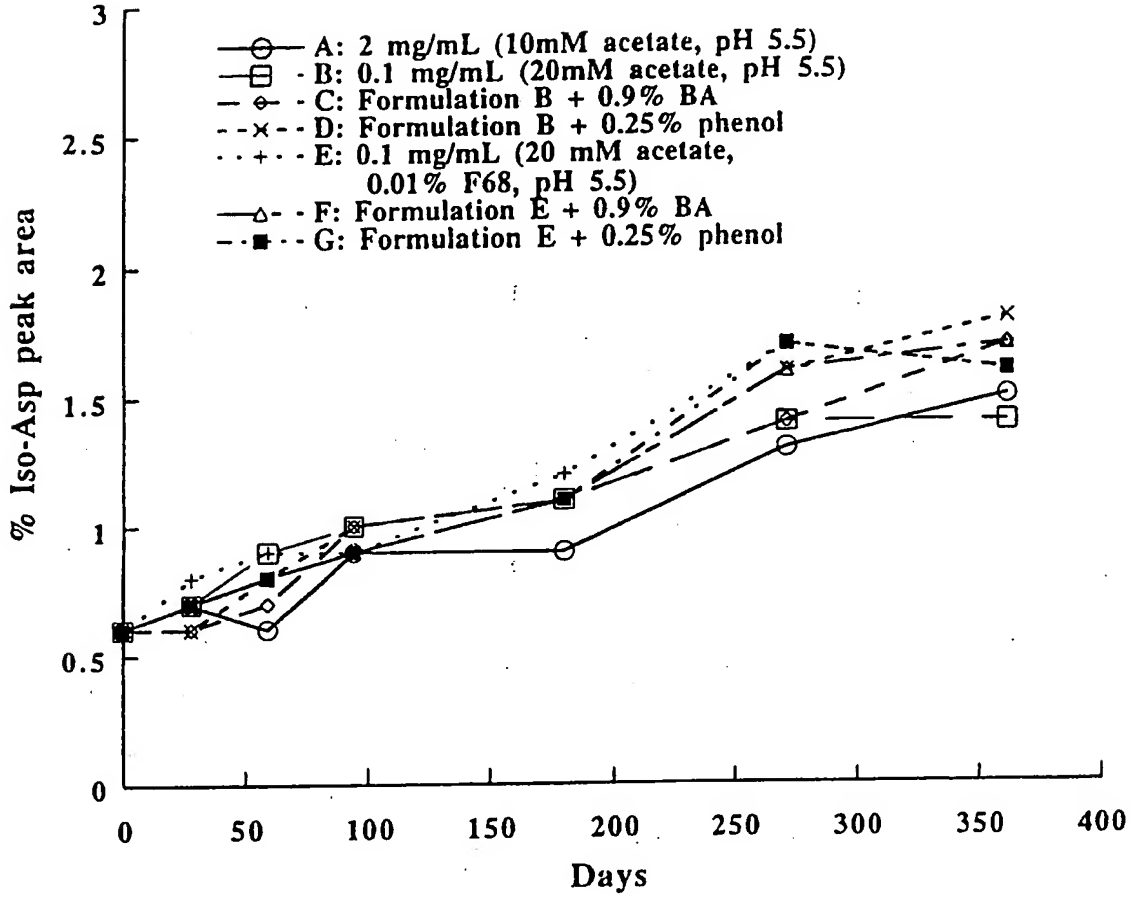

A: 2 mg/mL (10mM acetate, pH 5.5)
B: 0.1 mg/mL (20mM acetate, pH 5.5)
C: Formulation B + 0.9% BA
D: Formulation B + 0.25% phenol
E: 0.1 mg/mL (20 mM acetate, 0.01% F68, pH 5.5)
F: Formulation E + 0.9% BA
G: Formulation E + 0.25% phenol
% Iso-Asp peak area
Days
0
0.5
1
1.5
2
2.5
3
0
50
100
150
200
250
300
350
400

FIG. 11

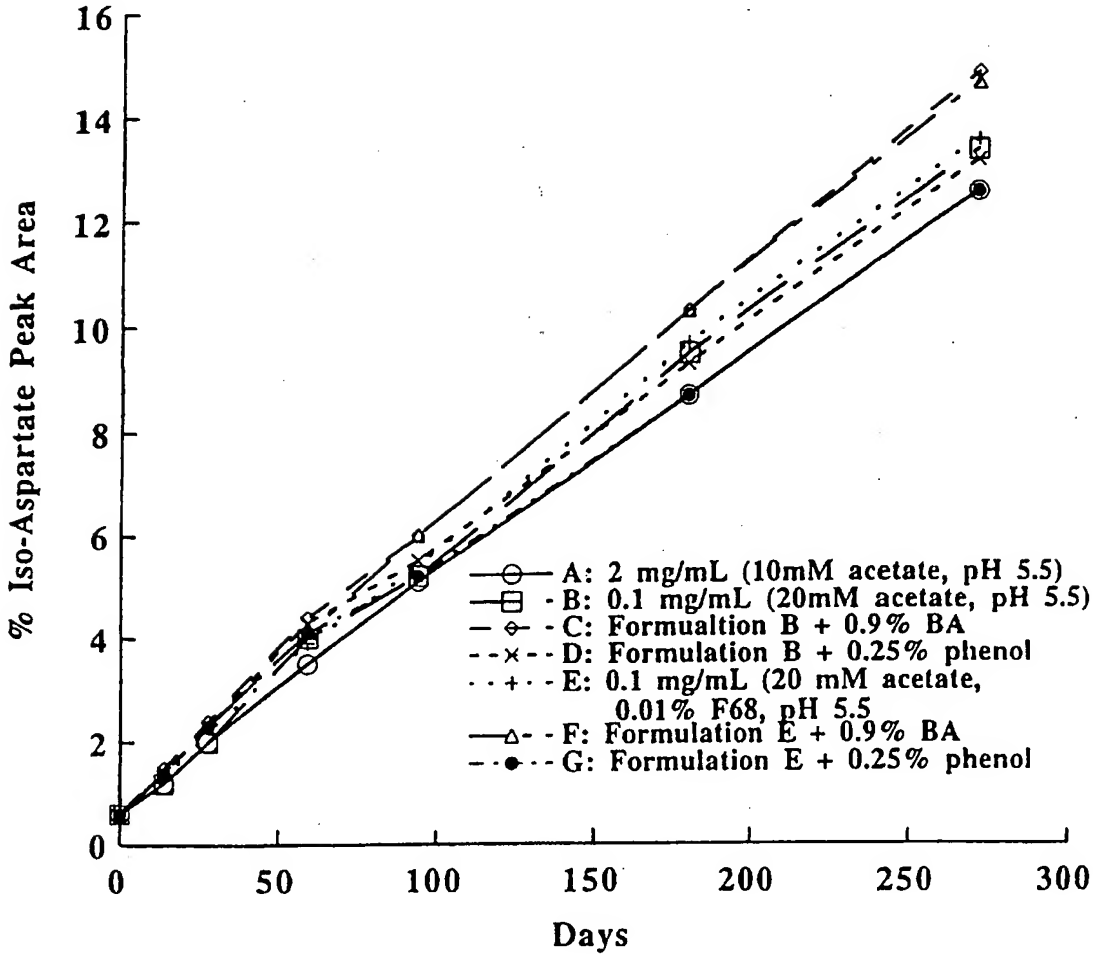
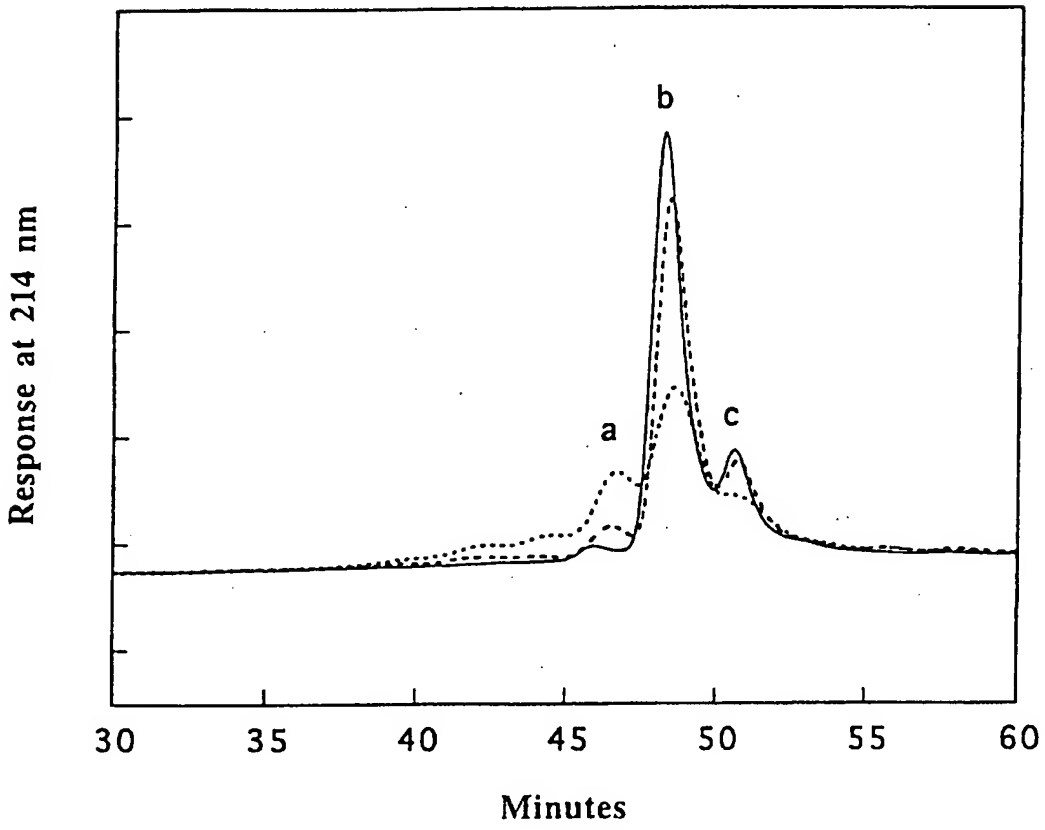

% Iso-Aspartate Peak Area
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
Days
A: 2 mg/mL (10mM acetate, pH 5.5)
B: 0.1 mg/mL (20mM acetate, pH 5.5)
C: Formualtion B + 0.9% BA
D: Formulation B + 0.25% phenol
E: 0.1 mg/mL (20 mM acetate, 0.01% F68, pH 5.5
F: Formulation E + 0.9% BA
G: Formulation E + 0.25% phenol

FIG. 12



a: Mono- & di-oxidized 118/118 and oxidized N-terminally clipped rhNGF
b: 118/118 rhNGF homodimer
c: Ser-Gly 118/118 rhNGF (1-chain)

FIG. 13

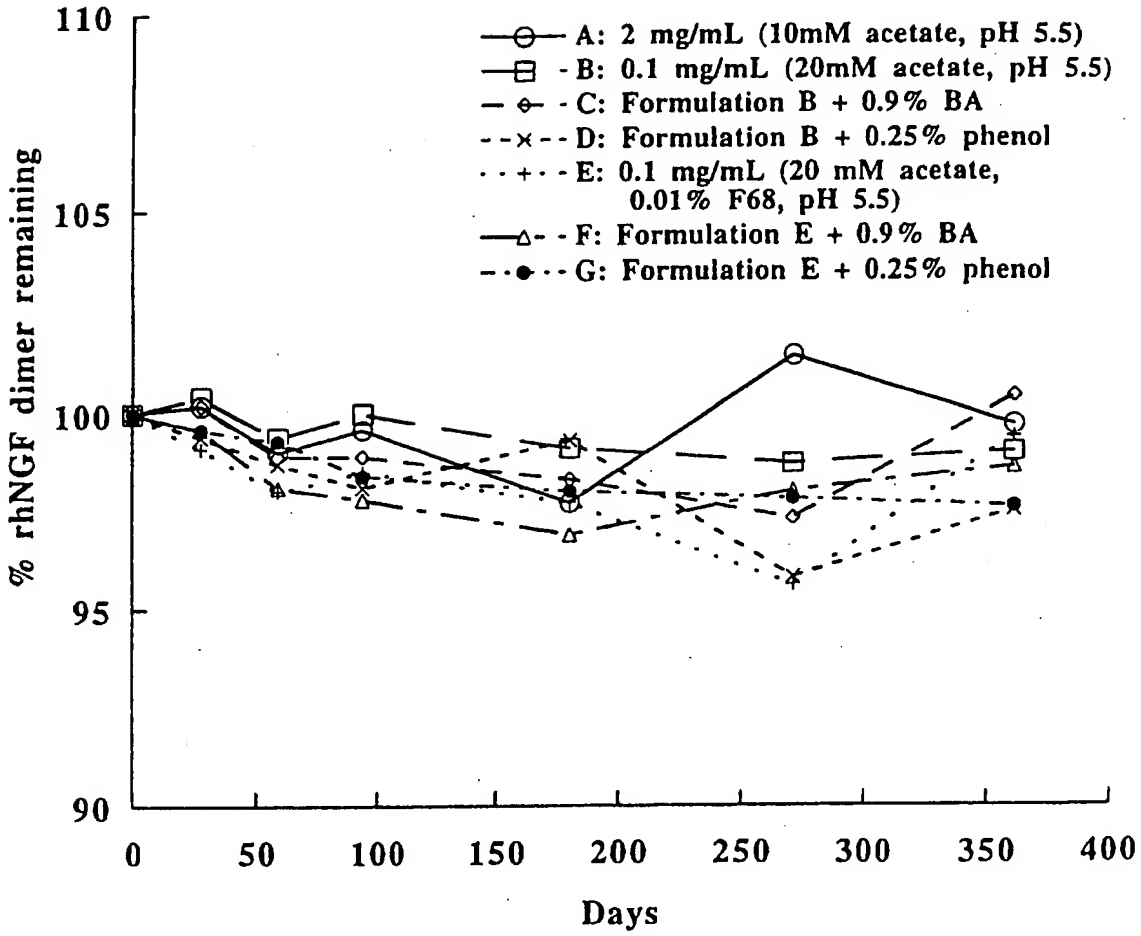

110
105
100
95
90
% rhNGF dimer remaining
0
50
100
150
200
250
300
350
400
Days
A: 2 mg/mL (10mM acetate, pH 5.5)
B: 0.1 mg/mL (20mM acetate, pH 5.5)
C: Formulation B + 0.9% BA
D: Formulation B + 0.25% phenol
E: 0.1 mg/mL (20 mM acetate, 0.01% F68, pH 5.5)
F: Formulation E + 0.9% BA
G: Formulation E + 0.25% phenol

FIG. 14

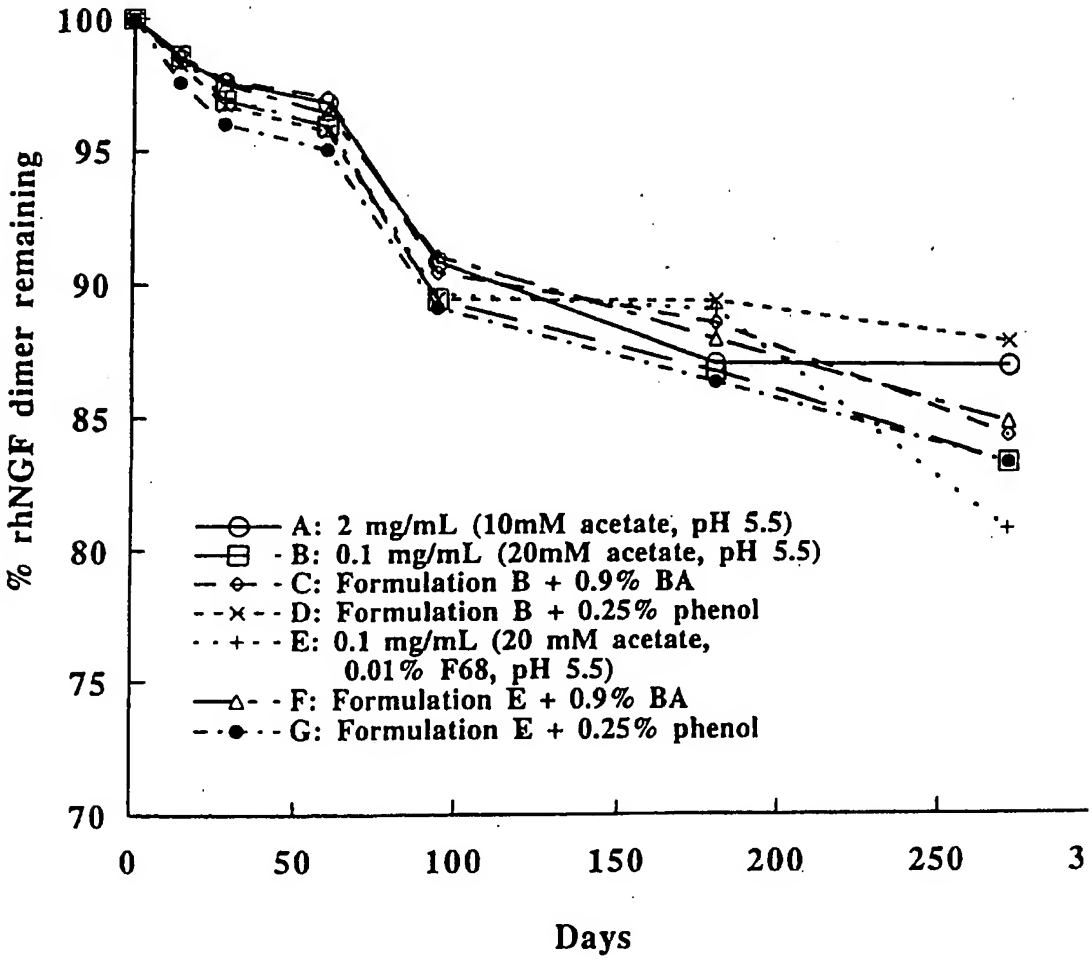

100
95
90
85
80
75
70
% rhNGF dimer remaining
0
50
100
150
200
250
3
Days
A: 2 mg/mL (10mM acetate, pH 5.5)
B: 0.1 mg/mL (20mM acetate, pH 5.5)
C: Formulation B + 0.9% BA
D: Formulation B + 0.25% phenol
E: 0.1 mg/mL (20 mM acetate, 0.01% F68, pH 5.5)
F: Formulation E + 0.9% BA
G: Formulation E + 0.25% phenol

FIG. 15

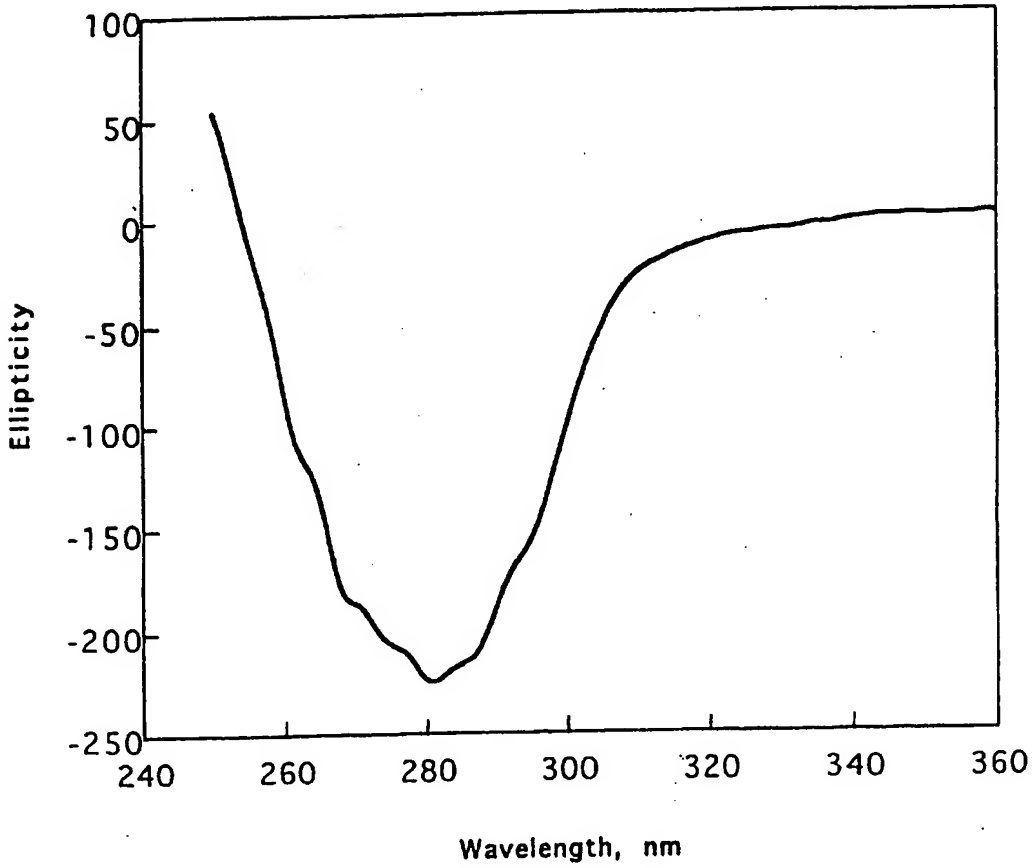

100
50
0
-50
-100
-150
-200
-250
Ellipticity
240
260
280
300
320
340
360
Wavelength, nm

FIG. 16

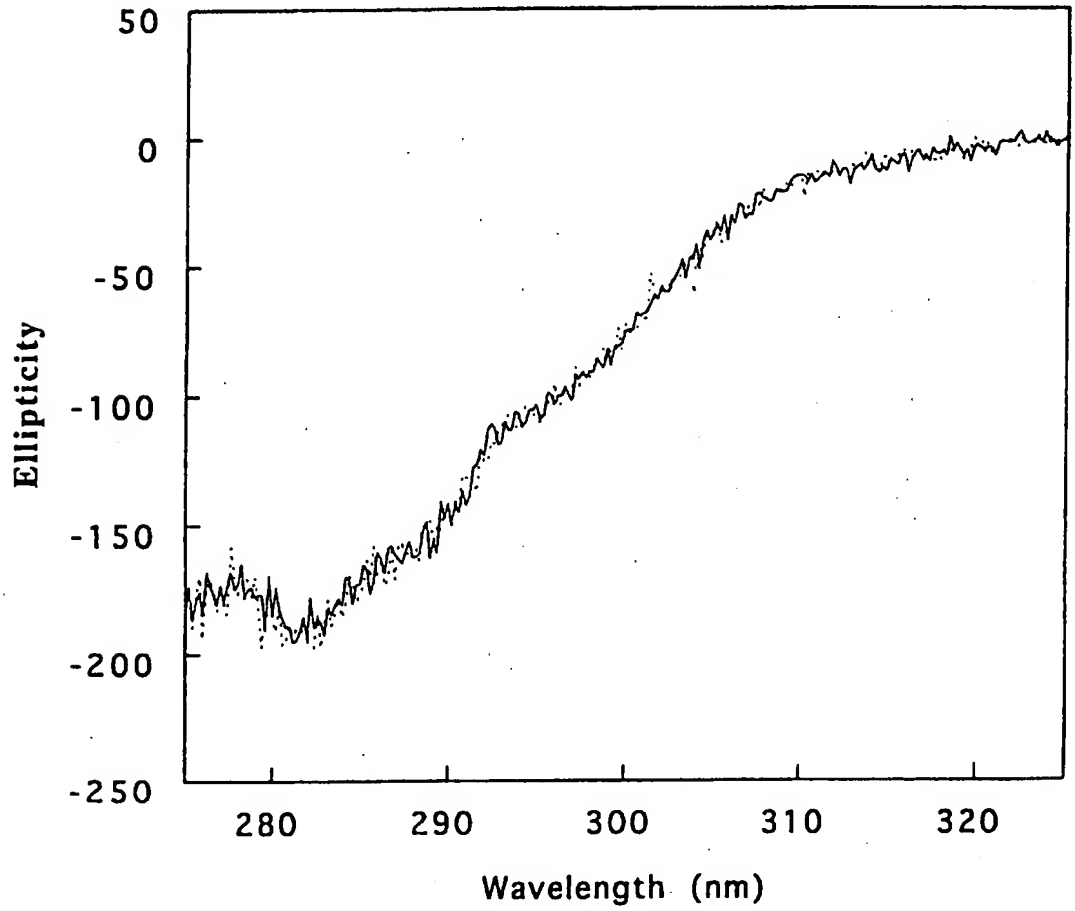

50
0
-50
-100
-150
-200
-250
Ellipticity
280
290
300
310
320
Wavelength (nm)

FIG. 17

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/16881

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18 A61K47/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 05845 A (SYNTEX-SYNERGEN NEUROSCIENCE JOINT VENTURE) 2 March 1995 see page 5, line 19 - line 28; claims	1-5,16, 18-24
Y	see page 5, line 34 - line 36 see page 6, line 3 - line 33 see page 9, line 6 - line 27	6-9, 12-15, 17,25-27
Y	WO 94 26302 A (GENENTECH, INC.) 24 November 1994 see page 5, line 17 - line 26; claims 1-15 see page 6, line 18 - page 7, line 23	6-9, 12-15,17

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1997	18.03.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Ryckebosch, A

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INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 96/16881

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOPHYSICAL JOURNAL, vol. 66, no. 2 PART 2, 1994, NEW YORK, N.Y., US, page A401 XP000645428 L.R. DE YOUNG ET AL.: "TEMPERATURE AND PH DEPENDENCE OF RECOMBINANT HUMAN NERVE GROWTH FACTOR DIMER DISSOCIATION." cited in the application see item Th-Pos247 -----	25-27

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr ·al Application No
PCT/US 96/16881

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9505845 A	02-03-95	AU 7566894 A	21-03-95
		BR 9407278 A	01-10-96
		CA 2169834 A	02-03-95
		CN 1133012 A	09-10-96
		CZ 9600424 A	15-05-96
		EP 0721343 A	17-07-96
		FI 960750 A	20-02-96
		LT 96011 A,B	25-07-96
		LV 11279 B	20-10-96
		NO 960651 A	19-02-96
		NZ 271873 A	28-10-96
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		SK 18396 A	01-10-96
		ZA 9406333 A	19-02-96
WO 9426302 A	24-11-94	AU 6782294 A	12-12-94
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		CN 1122575 A	15-05-96
		CZ 9502955 A	17-04-96
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